Regulation of HPV 16-E7 by E2, Phosphorylation and the Proteasome

Thesis

Regulation of HPV16-E7 by E2, Phosphorylation and the Proteasome

Noor Gammoh

This thesis is submitted for the degree of Doctor of Philosophy in the faculty of Life Sciences of the Open University, UK

International Centre for Genetic Engineering and Biotechnology (ICGEB),

Trieste – Italy

Director of Studies: Lawrence Banks, Ph.D

Second Supervisor: John Doorbar, Ph.D

September, 2007

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Contents

List of Figures

Abstract

In order to ensure a productive life cycle, Human Papillomaviruses (HPVs) require fine regulation of their gene products. Uncontrolled activity of the viral oncoproteins, E6 and E7, results in the immortalisation of the infected epithelial cells and thus prevents the production of mature virions. Here, we investigate the regulation of HPV-16 E7 activities through its interaction with both viral and cellular gene products. First, we show that HPV-16 E7 and E2 can interact directly and the region mediating this interaction is defined on each protein. The expression of E2 inhibits some of E7 oncogenic activities including primary cell transformation, induction of centrosome abnormalities and pRB degradation. In addition, E2 can stabilise E7 and redirect its localisation where it can associate with some of E2's activities such as transcriptional activation and mitotic chromosome binding. Secondly, we provide evidence that E7 can be phosphorylated by CDK2 *in vitro* preferentially on its N-terminal domain, and we hypothesise that this occurs on more than one residue on E7. *In vivo*, we show that the activity of CDK2, as well as CKII, is necessary for the stability of E7. Finally, we identified an interaction between HPV-16 E2 and E7 with the cellular oncoprotein, Mdm2. Mdm2 appears to destabilise E7 targeting it to proteasome-mediated degradation at PML bodies. The stability of E7 in cells that have reduced expression of Mdm2 is markedly increased indicating that the expression of Mdm2 indeed destabilises E7. In the case of the Mdm2 interaction with E2, we observe that E2 inhibits Mdm2 mediated degradation of p53 and pRB and that the expression of Mdm2 enhances E2's transcriptional activity and induces its re-localisation at specific structures within the nucleus. Overall, our findings expand our knowledge of the regulation of virally encoded proteins both through direct protein-protein interactions between themselves and through their interactions with cellular proteins.

Introduction

Cancer and Human Papillomaviruses (HPVs)

Cancer is a genetic disease that is mainly characterised by indefinite cell growth and division capacity (Vogelstein & Kinzler, 2004). Approximately 10 million new cancer cases are estimated to occur every year by the World Health Organisation. The development of cancer is a multi-step event and involves the deregulation of multiple cellular pathways which eventually leads to a wide range of molecular and clinical diagnoses. Despite the variability of the disease, malignant cells appear to share a number of physiological features (reviewed by Hanahan & Weinberg, 2000). These include undisturbed cell growth, deregulation of apoptotic machinery, limitless replication capacity and metastatic tendency. In addition, most cancer cells have abnormalities in their genomes and share common deregulated molecular pathways, for example the pRB and p53 pathways, which have been highlighted as major targets of all DNA tumour viruses (Klein, 2002).

A number of factors trigger the development of cancer, and when identified, these can serve as tools to understand cancer progression and, most importantly, for the development of therapy. Up to 15% of human cancers are associated with viruses (Gatza et al, 2005). One example of this is cervical cancer; the second largest cause of cancer deaths in women with an estimated half a million new cases in the year 2002 half of which were fatal (Parkin, 2006). Infection with Human Papillomavirus (HPVs) has been detected in virtually all cervical cancer cases. HPVs can also infect other epithelial sites and are thus linked to a number of clinical outcomes including benign warts of the skin, non-melanoma skin cancer in inherited epidermodysplasia

verruciformis patients and a number of head and neck cancers (Gillison & Shah, 2001). In the cervix, infection with only certain types of papillomaviruses, termed high-risk HPVs (such as HPV-16, -18 and -31), can contribute to the development of cancer. Their low-risk counterparts (such as HPV-6 and -11) also infect the mucosal epithelium and induce the development of genital warts; however they are rarely associated with malignant lesions.

The development of cervical cancer in the absence of HPV infection is extremely rare. Human cervical carcinoma cell lines that are HPV-negative were found to harbour mutations in both *p53* and *pRB* sequences, the major targets of high-risk HPV oncoproteins E6 and E7, respectively (Scheffner et al, 1991). In contrast, *p53* and *pRB* are invariably wild-type in HPV-positive cells, indicating the importance of disrupting these pathways in cervical carcinogenesis as well as explaining the rarity of non-HPV induced cervical cancers. The expression of E6 and E7 directly contributes to the progression of cervical cancer in HPV infections. E6 and E7 support the viral life cycle by providing a cellular environment conducive to viral replication. Both proteins have multiple cellular binding targets (Figure 1) and act cooperatively to induce cell cycle progression and inhibit apoptosis, mainly by disrupting the p53 and pRB pathways (Munger et al, 2004). However, their uncontrolled expression is directly associated with the development of cervical abnormalities and, ultimately, malignant progression. Experimentally, the combined activities of E6 and E7 induce the development of tumours in mice and the immortalisation of primary human keratinocytes (Matlashewski et al, 1987; Riley et al, 2003). Furthermore, in HPV-positive cell lines, inhibiting the expression of both proteins either transcriptionally, by the viral transcriptional regulator E2, or post-transcriptionally, using RNA interference (RNAi)

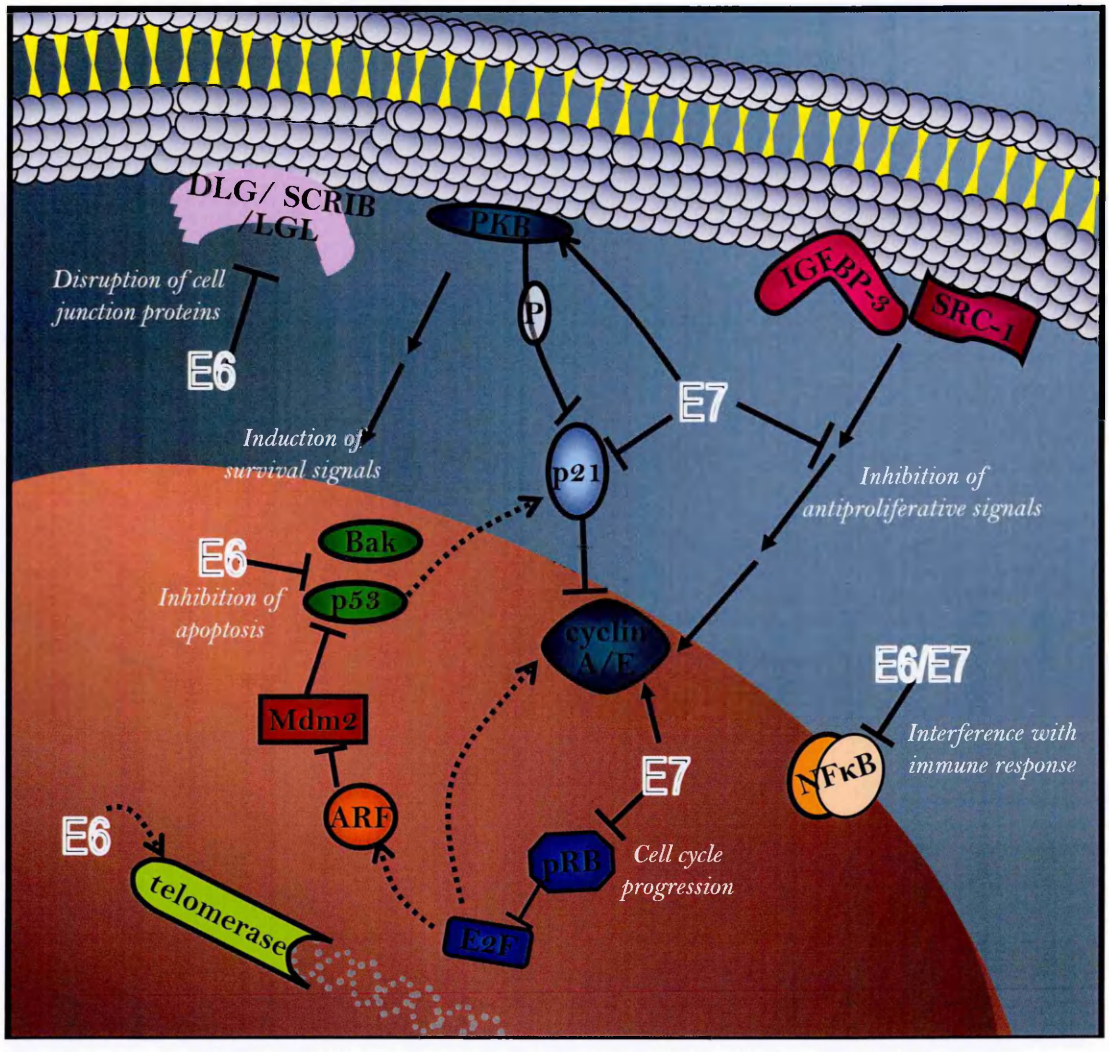


Figure 1. Cellular pathways disrupted by high-risk HPV E6 and E7. Arrows connect various proteins in a pathway, where pointed ends indicate activation, blunt ends indicate inhibition of activity and dashed lines indicate transcriptional regulation.

technology, results in the inhibition of cell growth and the induction of apoptosis (Dowhanick et al, 1995; Yoshinouchi et al, 2003).

The phenotypic effects induced by the HPV oncoproteins on the cell overlap with some of the major physiological characteristics of a cancerous cell (Table 1) (as reviewed by Hanahan & Weinberg, 2000). The E7 protein induces cellular proliferation by degrading pRb and by disrupting the activity of cyclin-dependent kinase inhibitors, p21 and p27, thereby activating transcription factors required for G1/S-phase progression (Dyson et al, 1989; Funk et al, 1997). E7 can also directly abrogate normal centrosome replication which can result in increased genetic abnormalities and genome amplification (Duensing & Munger, 2003). On the other hand, E6 plays a role in circumventing apoptotic pathways induced by the activities of E7 through the degradation of key pro-apoptotic proteins, such as p53 and Bak (Scheffner et al, 1990; Thomas & Banks, 1998). E6 also transcriptionally upregulates hTERT, the catalytic subunit of telomerase, thereby ensuring increased replicative competence of the cell by maintaining chromosome ends (Klingelhutz et al, 1996). Moreover, both E6 and E7 can modulate cell signalling cascades by desensitising cells to growth-inhibitory factors, such as TGF-beta and IGFBP-3 (Favre-Bonvin et al, 2005; Mannhardt et al, 2000), and by upregulating stimulatory growth signals, such as the PKB pathway, resulting in increased expression of proliferatory genes even in the absence of growth factors (Pim et al, 2005). As cells progress into a tumorigenic state, they lose cell-cell contact and thus attain a state of uncontrolled cell division and high mobility. E6 is thought to contribute to this stage of cancer development by degrading certain PDZ-containing proteins which are components of cell junctions, such as Scribble, Dlg and MAGI (Massimi et al, 2004).

Table 1: Various pathways regulated by high-risk HPV oncoproteins; E6 and E7.			
Cancer cell phenotype	**cellular pathway involved**	**High-risk viral protein action**	**Reference**
Deregulation of apoptosis	p53 and Bak pro-apoptotic pathways	E6 induces degradation of both p53 and Bak	(Scheffner et al, 1990; Thomas & Banks, 1998)
Undisrupted cell growth	pRB pathway	E7 induces degradation of pRB, and other pocket proteins	(Boyer et al, 1996)
Auto-regulated growth signalling	PKB signalling cascade	16E7 appears to upregulate PKB pathway	(Menges et al, 2006; Pim et al, 2005)
Renewable replicative capacity	Telomerase activity	E6 increases hTERT transcription	(Klingelhutz et al, 1996)
Metastasis	Disruption of cell-cell junction	Possible involvement of PDZ-containing proteins-degradation by E6	(Massimi et al, 2004; Simonson et al, 2005)

HPV infection is essential but insufficient

The majority of HPV infections are immunologically cleared from the infected individual (Jenson et al, 1991); in cases of persistent viral infection there is a long latency period of many years between initial infection with HPV and the development of premalignant lesions. In addition, exposure to external stimuli, such as oestrogens or UV radiation, is required to promote tumour progression induced by HPV oncoproteins in animal models. These two observations suggest the contribution of additional factors to the development of HPV-induced malignancies (Bosch et al, 2002). These may include unidentified hereditary risk factors (Hemminki et al, 1999; Magnusson et al, 1999), association with other diseases such as epidermodysplasia verruciformis (Harwood & Proby, 2002) and immune suppression (Palefsky, 2006; Petry et al, 1994). Other environmental cofactors include the prolonged use of hormonal contraceptives, high parity (full term pregnancy) and cigarette smoking (Bosch et al, 2006).

The viral life cycle

The Virus

Papillomaviruses belong to the *Papillomaviridae* family, which together with other viruses such as Herpesviruses and Adenoviruses are classified as DNA tumour viruses. To date, more than 100 different subtypes of papillomaviruses have been identified, varying in their host species, sites of infection and clinical diagnosis. All papillomavirus genomes consist of a double-stranded circular DNA molecule, around 7.9Kb in size, contained within the viral capsid (Zheng & Baker, 2006). The viral genome can be divided into three main regions; non-coding, early-coding and late-coding (Figure 2). The non-coding region (Long Control Region, LCR, or Upper Regulatory Region, URR) contains an origin of DNA replication and a number of transcription regulatory sites. Viral non-structural proteins (E1, E2, E4, E5, E6 and E7) are encoded by the early-coding region, meanwhile structural proteins (L1 and L2) are encoded by the late-coding region. The additive size of the virus open reading frames plus the LCR exceeds the total size of the viral genome and therefore a number of coding sequences are overlapping. In addition, virally encoded transcripts undergo multiple splicing events which expands the potential number of viral gene products (Zheng & Baker, 2006).

The association between papillomavirus life cycle and its host

Papillomaviruses are strictly epitheliotropic and this tissue tropism could be, in part, due to the expression of certain epithelia-specific transcriptional factors which are essential for viral gene expression (Bedrosian & Bastia, 1990). These include

KRF-1 (Mack & Laimins, 1991), Epoc-1 (Yukawa et al, 1996) and PEF-1 (Fergusson & Campo, 1998). In addition, papillomavirus infection is intimately associated with the differentiation of its host tissue. Viral gene expression is regulated differentially through the life cycle of the infected tissue, which influences viral genome amplification and the production of viral particles (Doorbar, 2005).

The normal life of the epithelial cells involves the progression of actively dividing basal stem cells into a terminally differentiated, cell cycle arrested and division-incompetent state. In the upper layers of the epithelial strata, cells eventually undergo nuclear breakdown and keratin accumulation, and are shed into the surrounding environment. The association between the virus life cycle and its host cell differentiation program is highly significant since papillomaviruses are non-lytic and the release of viral particles ultimately depends on the shedding of epithelial squamous cells from the upper layers of the cutaneous or mucosal epithelium (Figure 3a). Papillomaviruses, however, express proteins which can delay the cell cycle exit of differentiating epithelial cells and thereby ensure the prolonged maintenance of a cellular environment that can support viral genome replication.

The papillomavirus life cycle and its possible consequence of cancer progression are discussed below. There are considerable differences from one virus type to another in the actual sequence of events: here the main focus will be on the life cycle of high-risk HPV types (e.g. HPV-16, -18 and -31).

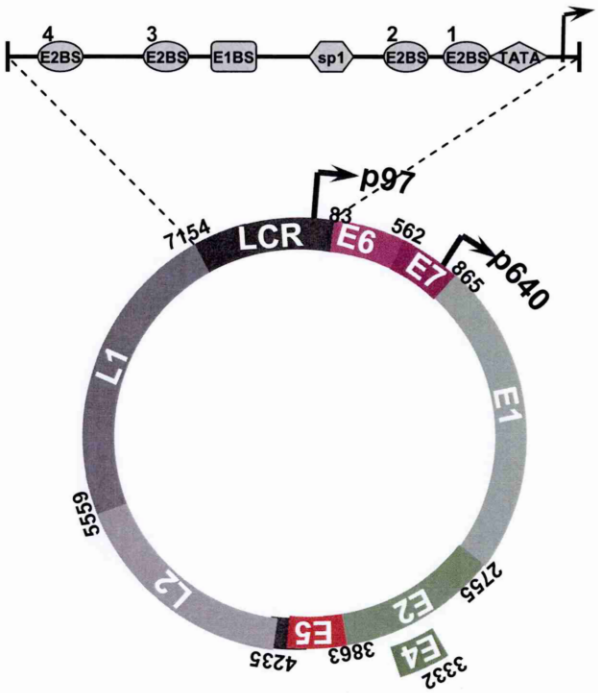


Figure 2. Organisation of HPV-16 genome. The six early coding sequences (E1-E7), the late coding sequences (L1 and L2) and the LCR are shown. Numbers indicate the nucleotide at which each functional sequence starts (adopted from NCBI; GenBank accession number NC_001526). The early (p97) and late (p640) promoters are represented by arrows. A magnification of the LCR shows the various E2 binding sites (E2BS), numbered from 1 to 4, as well as the E1 (E1BS), sp1 and TATA-box binding sites.

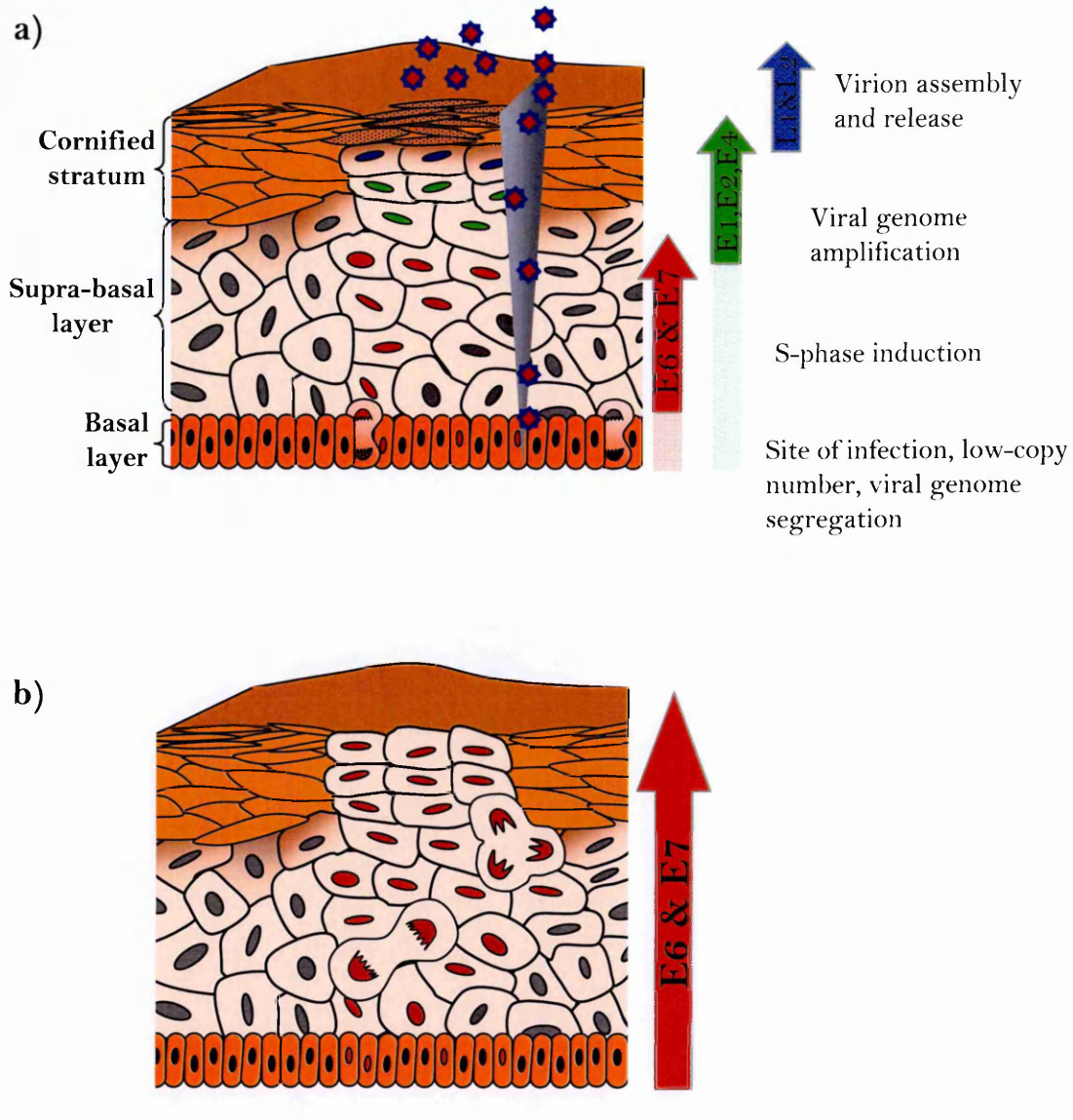


Figure 3. HPV life cycle. a) A model of the epithelium is shown here along with the HPV life cycle. HPV gain access to the basal cells through micro-traumas. As these cells divide, one daughter cell starts to migrate to the supra-basal layer while another remains undifferentiated. By segregating their genomes equally in mitosis, HPV can both maintain viral infection in the basal cells and progress into the upper epithelial layers where viral genome amplification (green), virion assembly (blue) and release (brown dotted blue) take place. The expression of E6 and E7 is important for S-phase induction (red) in terminally differentiated cells (grey). b) HPV-induced malignancies. Continual expression of E6 and E7 and loss of expression of other viral proteins. The normal differentiation of the epithelium is disrupted.

Infection and Early Stages (E1 and E2)

Initial infection of papillomaviruses probably occurs through microtraumas in the upper layers of the epithelia which provide the virus with access to lower basal cells. The viral structural proteins, L1 and L2, mediate virion uptake into the cell by endocytosis and viral genome entry into the nucleus (Day et al, 2003). In these cells, viral episomes are maintained at a low copy number of between 10-200 copies per cell (De Geest et al, 1993; Stanley et al, 1989). The virally expressed helicase E1 plus the transcriptional activator E2 are thought to be required at this stage of the viral infection. Both E1 and E2 are important for the replication of viral episomes and E2 is additionally involved in the control of viral gene expression and genome segregation.

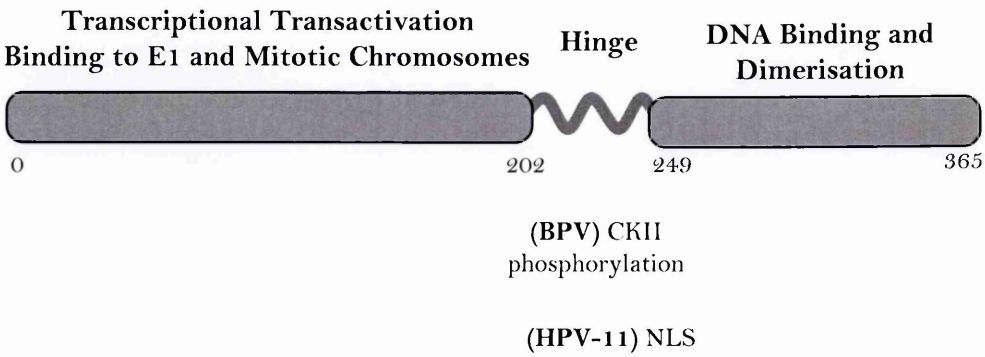
E2 is a nuclear protein of around 45 kDa in size and consists of a transactivation domain in the N-terminal half of the protein, a middle non-conserved hinge region and a C-terminal domain that mediates DNA binding and protein dimerisation (Figure 4a). Alternative splicing of the E2 protein can also produce shorter forms that lack the N-terminal region. Full length E2 binds as a homodimer specifically to a consensus palindromic sequence, $ACCN_6GGT$, through its C-terminal DNA binding domain (Androphy et al, 1987). Several E2-binding sites are present in the LCR of the viral genome, ranging from 4 sites in HPV up to 17 sites in Bovine Papillomavirus Virus (BPV). In HPV genomes, two E2-binding sites flank the viral origin of replication (ori), a third site lies directly upstream of the early promoter (p97 in HPV-16 and p105 in HPV-18) which controls the expression of E6 and E7 (Rohlfs et al, 1991; Sang & Barbosa, 1992), and a fourth site lies at the 5' end of the LCR (Figure 2b). Binding of E2 to the LCR results in a structural bend of the DNA

helices and facilitates the binding of both a number of transcription factors for viral gene expression and the E1 helicase to the viral ori for the initiation of DNA replication (Thain et al, 1997).

In addition to the E2 binding sites, viral gene expression is regulated through multiple regions within the LCR including the TATA box and sp1 binding site (Gloss & Bernard, 1990). The binding of E2 proteins from HPV-16 and -18 was shown to both activate and repress expression of viral genes from the early promoter in a dose-dependent manner (Bouvard et al, 1994b). E2 binds with the highest affinity to binding site number four in the LCR (Figure 2) through which it can activate transcription of the early promoter (Steger & Corbach, 1997). At high levels of E2, it is thought that transcriptional repression takes place as E2 occupies all of its four binding sites. This results in the displacement of TATA-Binding Protein (TBP) and sp1, both of which are required for transcriptional activation, from their binding sites in the LCR (Dong et al, 1994; Tan et al, 1992). Furthermore, methylation of the E2 binding sites in the LCR, which occurs differentially through the life cycle of the virus, inhibits the binding of E2 to the viral early promoter and can further regulate viral gene expression (Kim et al, 2003).

The activities of E2 may be regulated through sequences within its hinge region. In BPV E2, a Casein Kinase II (CKII) phosphorylation site has been identified at serine 301 which lies within the hinge region (Penrose et al, 2004). Phosphorylation of this site results in a structural change of the E2 protein and increases its proteasome-mediated degradation. Mutations of this serine in BPV-1 E2 result in a more stable E2 protein and consequently an increase in the viral

a)



b)

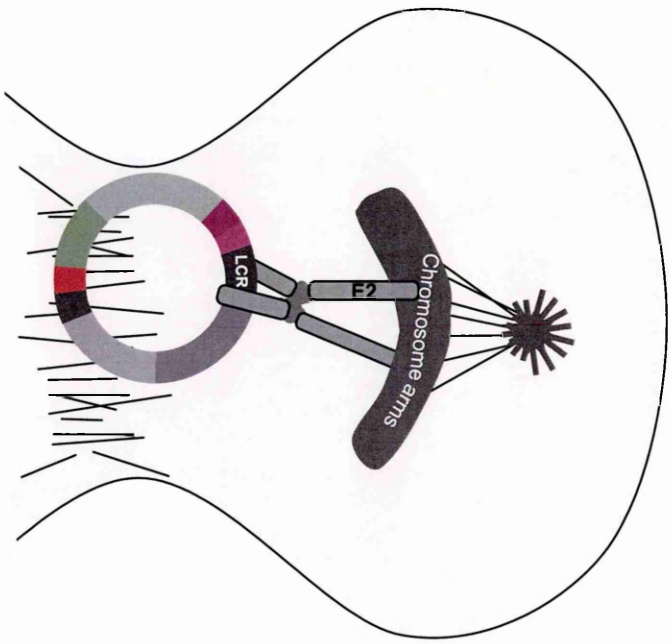


Figure 4. The E2 protein. a) A cartoon depicting the main domains of HPV-16 E2 protein including the transcriptional transactivation domain and the E1 and chromosome binding region at the N-terminal half, the middle flexible hinge region and the DNA binding and dimerisation domain at the C-terminal half. Additional sites that are identified on other E2 proteins are shown underneath. b) Viral genome segregation mediated by E2 which binds as a dimer to the viral genome through its N-terminal end and to mitotic chromosomes through its C-terminal end.

DNA copy number. The CKII consensus sequence is not conserved in HPV E2 proteins; however, HPV-18 E2 is also degraded through the proteasome but this seems to involve sequences within its N-terminal domain (Bellanger et al, 2001). In addition, the localisation of E2 in or out of the nucleus could serve as an additional mechanism by which its activities can be controlled. The nucleus is the main cellular compartment where E2 can exert its major activities. Nuclear localisation of proteins can be controlled through the presence of nuclear localisation or nuclear export sequences (NLS or NES, respectively). In HPV-11 E2, an NLS was identified in the hinge region (Zou et al, 2000), meanwhile in HPV-16 E2 the NLS is present in the C-terminal domain (Klucevsek et al, 2007). HPV-18 E2 additionally contains an NES in its N-terminal domain which, through an unknown mechanism, permits the shuttling of the protein in and out of the nucleus (Blachon et al, 2005).

Viral genome replication

In the infected basal cells, E2 plays a primary role in viral genome replication by recruiting the viral helicase, E1, onto the origin of replication (Mohr et al, 1990). E1 is 73 kDa in size, it possesses both helicase and ATPase activities and is important for both the initiation and the progression of viral DNA replication. The N-terminal end of E1 mediates both its DNA binding and nuclear localisation, while ATPase/Helicase activities, oligomerisation, DNA polymerase α and E2 binding are attributed to the C-terminal end. On its own, E1 has a low affinity for binding DNA, whereas in the presence of E2 this is greatly increased. Due to the highly conserved E2 DNA recognition sequence, E1 and E2 proteins from one

virus type can mediate the replication of the viral genome from a different papillomavirus type. However, mixtures of the E1 and E2 proteins from two different HPVs mediate genome replication much less efficiently than when E1 and E2 are from the same HPV type, suggesting a degree of conservation in the E1/E2 interaction (Gopalakrishnan et al, 1999; Zou et al, 1998). This specificity is determined by the E1 protein and, specifically, by its ATPase domain. Thus, replacing just the ATPase domain of HPV-16 E1 with the ATPase domain of HPV-11 E1 restores its cooperative DNA replication activity with HPV-11 E2 (Zou et al, 1998).

After being recruited to the ori by E2, further E1 molecules are recruited to form dihexameric structures on the DNA. This process requires ATP hydrolysis (Sanders & Stenlund, 1998) and its disruption interferes severely with DNA replication (Schuck & Stenlund, 2005). To allow the replication forks to initiate, E2 must first be displaced from the ori. This takes place in an ATP-dependent manner through the combined activities of the E1 helicase (Sanders & Stenlund, 1998), cellular heat shock proteins (HSP70 and HSP40) (Lin et al, 2002) and binding of E1 to topoisomerase1 (Conger et al, 1999; Masterson et al, 1998). The assembly of E1 complexes on DNA causes major structural changes in the DNA, resulting in DNA melting (Chen & Stenlund, 2002). Furthermore, the binding between E1 and the primase subunit of DNA polymerase α is important for recruitment of the cellular DNA replication machinery to the viral origin (Masterson et al, 1998). DNA unwinding then proceeds, which requires topoisomerase1, single stranded DNA binding protein (RPA) and ATP (reviewed by Stenlund, 2003). Replication is then thought to proceed in a bi-directional theta mode, maintaining viral genomes as low copy number episomes in the basal layer (Flores & Lambert, 1997).

A direct role for other viral proteins in viral genome replication can be postulated. The p53 protein can act as an inhibitor of viral DNA replication; this inhibition involves the DNA binding domain of p53 but not the transactivating domain (i.e. excludes its apoptotic and cell arrest functions) (Lee & Laimins, 2004; Lepik et al, 1998; Massimi et al, 1999; Thomas et al, 1999b). The activity of E6 in inducing the degradation of p53 is well documented and it may thus enhance viral DNA replication. In addition, E7 might play a role in increasing the activity of E1 by upregulating CDK2/cyclin E activity (see below). CDK2/cyclin E-mediated phosphorylation of E1 is known to be important for viral replication (Lin et al, 2000; Ma et al, 1999) since this regulates E1 localisation in the nucleus (Deng et al, 2004).

Viral genome segregation

As basal stem cells divide, one daughter cell migrates into the upper epithelial cell layer while the other remains as a reserve stem cell (Watt, 1998). In the case of papillomavirus infection, the viral episomes are required to segregate equally upon basal cell division to ensure both genome maintenance in the epidermal stem cells and a productive viral life cycle in the upper epithelial layers. One way of doing this would be to segregate the viral genomes using chromosome arms as vehicles (McBride et al, 2004; McBride et al, 2006). The phenomenon of viral genome segregation is shared by a number of viruses but the mechanism appears to be not conserved, neither between different viruses nor even within different viral subtypes (Oliveira et al, 2006). Epstein-Barr Virus (EBV), Human Herpes Virus (HHV) and Herpesvirus Saimiri (HVS) each encode proteins which mediate the

binding of their viral episomes to various cellular targets on mitotic chromosomes (Table 2). Such redundancy in the utilisation of genome segregation strategies of very different viruses may highlight the essential role of this process for the completion of the viral life cycle.

The first evidence that a papillomavirus-encoded protein can associate with mitotic chromosomes came from a study of BPV E2 (Skiadopoulos & McBride, 1998). This was based on previous work showing that E2 is required for the long-term maintenance of minichromosomes containing E2 binding sites in a mode that is independent of its replication activity (Piirsoo et al, 1996). BPV-1 E2 was shown to mediate episome segregation by acting as a bridge between viral genomes and dividing chromosomes (Figure 4b). This is achieved through the C-terminal DNA binding domain of E2 attaching to the viral DNA, whilst at the same time the N-terminal domain interacts with the chromatin-binding and bromodomain-containing protein, Brd4 (McPhillips et al, 2005; You et al, 2004). Brd4 is a nuclear protein that attaches to acetylated chromatin during interphase and mitosis and plays a role in regulating transcription and cell growth (Dey et al, 2003). Although E2 proteins from a number of HPV types can bind to Brd4, it is still not certain whether Brd4 is essential for their viral genome segregation (Abbate et al, 2006; McPhillips et al, 2006). One study has shown that this interaction seems to be more relevant for HPV E2 mediated transcription rather than chromosome localisation (McPhillips et al, 2006). Further studies have highlighted alternative ways by which HPV E2 proteins may mediate episome segregation. Detailed fluorescence microscopy has shown that E2 from the low-risk HPV-11, and possibly other HPV types, associates with the mitotic spindle apparatus rather than the chromosome arms during mitosis (Dao et al, 2006; Van Tine et al, 2004). Other

studies propose alternative cellular proteins, such as mitotic kinesin-like protein 2 (MKlp2) (Yu et al, 2007) and ChIR (Parish et al, 2006), as possible mediators of HPV E2 interaction with mitotic chromosomes. Such variations between different studies might be due to differences in experimental procedures. Alternatively, given the example of the LANA protein of HHV8, which uses a number of different cellular proteins to mediate its episome segregation (Krithivas et al, 2002), it is possible that the equivalent HPV E2 function is also mediated by more than one cellular protein. Therefore, the effects of disturbing the interaction between E2 and one protein partner might actually be masked by the interaction of alternative cellular partners, indicating a zdegree of redundancy.

Table 2: Various virally encoded proteins that are involved in viral DNA segregation

Virus	Tethering protein	Cellular target	Reference
Human Papillomavirus (HPV)	E2	Brd4 (possibly additional targets)	(You et al, 2004)
Epstein-Barr Virus (EBV)	EB-nuclear antigen-1 (EBNA-1)	P40 (nucleolar proliferation antigen	(Wu et al, 2000)
Human Herpes Virus 8 (HHV8)	Latency-associated nuclear antigen (LANA)	Histone H1, Dek, MeCBP, Brd2, H2A-H2B	(Barbera et al, 2006; Krithivas et al, 2002; Viejo-Borbolla et al, 2005)
Herpesvirus saimiri (HVS)	ORF73	?	(Calderwood et al, 2004)

Lower Epithelial layer and Proliferative stage (E5, E6 & E7)

As the infected basal cell layers of the epithelia start to differentiate, their replicative capacity is greatly reduced (Watt et al, 2006). In these cells viral episomes are maintained at high copy number, and a replication switch into rolling circle mode is observed (Flores et al, 2000). In this phase, the E6 promoter is activated, which results in the enhanced expression of E1, E2, E5, E6 and E7. Three early gene products, E5, E6 and E7, possess proliferation stimulatory effects

which contribute to restoring the replicative capacity of the differentiated cells. E6 and E7 are expressed from a bicistronic mRNA (Tan et al, 1994) and their cooperative function in stimulating cell cycle progression is the most studied.

E7 is a small acidic phosphoprotein, with an isoelectric point of about 4.0, and shares some sequence homology with the Adenovirus E1a protein and the simian virus 40 large T antigen (LT) (Chellappan et al, 1992; Phelps et al, 1988). E7 is 98 amino acids in length (predicted molecular weight of 11kDa) and contains a zinc-binding domain in the C-terminal region (Rawls et al, 1990) whose structural integrity is necessary for the activity of E7 (McIntyre et al, 1993). High-risk HPV E7 has multiple cellular binding partners and can interfere with multiple cellular pathways. The main activity of E7 is to bind and inactivate the pocket protein family members; pRB, p107, and p130, and thus, by activating E2F transcription factors, it induces S-phase progression and restores the DNA synthesis machinery necessary for viral replication, (reviewed by Felsani et al, 2006). E7 also regulates cellular transcription by binding to the TBP (Massimi et al, 1997), TBP-associated factors (Mazzarelli et al, 1995) and members of the AP-1 transcription factor family such as c-Jun (Antinore et al, 1996). In addition, E7 stimulates cell cycle progression and transcription by inhibiting the activity of the cyclin-dependent kinase inhibitors p21 and p27 (Funk et al, 1997) and histone deacetylases (Brehm et al, 1999). Furthermore, cell cycle arrest induced by growth inhibitory signals, by DNA damage or by serum deprivation can all be abrogated by the activities of E7 (Demers et al, 1996).

The expression of E7 is critical for the viral life cycle: engineered viral genomes with disrupted expression of the E7 coding sequence do not support a productive

life cycle (Flores et al, 2000; Oh et al, 2004b; Thomas et al, 1999a). The specific importance of each of the above interactions of E7 in the context of the viral life cycle is, up to this point, largely unknown. In a recent study, the activity of wild-type E7 protein was monitored in a mutant pRB background that is defective in binding E7 but in which it retains its other activities (Balsitis et al, 2005). This study has shown that the interaction between E7 and pRB is important for the induction of DNA synthesis and for overcoming DNA damage-induced cell cycle arrest, therefore the disruption of pRB function by E7 is crucial for providing the cellular environment important for viral genome amplification. In addition to its function in controlling G1/S-phase progression, pRB also plays a role in cellular differentiation (Nguyen et al, 2004; Nguyen & McCance, 2005). Thus, E7 targeted degradation of pRB may also play a role in delaying the differentiation of infected epithelial cells and thereby extending the period during which viral replication can take place.

E7 exhibits pro-apoptotic activities on the cell (Kaznelson et al, 2004; Stoppler et al, 1998). The degradation of pRB and unscheduled DNA synthesis triggered by E7 may lead to an increase in p53 levels in the cell. One way in which this may occur is through E2F-mediated upregulation of the expression of p14ARF which acts as an inhibitor of Mdm2, and thereby inhibits Mdm2-mediated degradation of p53. Indeed, cells expressing E7 have elevated p53 levels (Demers et al, 1994; Jones & Munger, 1997) which is transcriptionally active as detected by the increased expression of p53 regulated genes, such as p21 (Seavey et al, 1999) and Mdm2 (Thomas & Laimins, 1998). In these cells, however, the activity of p21 in mediating cell cycle arrest is inhibited (see below) and the Mdm2-p53 binding is disrupted (Seavey et al, 1999).

E6 plays an important role in preventing the p53-mediated apoptosis induced as a result of E7's activities. The E6 protein is around 150 amino acids in length and contains two zinc binding fingers that are structurally similar to that found in E7. By binding to a HECT domain-containing ubiquitin ligase, named E6-associated protein (E6-AP), E6 enhances the targeting of p53 for proteasome-mediated degradation (Scheffner et al, 1993). The normal cellular targets of E6-AP are largely unknown, but mutations that disrupt its ubiquitin ligase activities or chromosomal deletions in its coding sequence are associated with the neurological disorder Angelman syndrome (Cooper et al, 2004). Viral genomes with complete disruption of E6 expression or which harbour mutations in the p53 binding region of E6 fail to maintain viral episomes (Park & Androphy, 2002; Thomas et al, 1999a). However, the episomal maintenance of viral genomes that harbour mutants of E6 defective in p53 binding can be rescued upon the disruption of the E7-pRB interaction, thus indicating the importance of the balanced activities of both oncoproteins (Park & Androphy, 2002). This rescue is only partial with respect to wild-type genomes, therefore full activities of both E6 and E7 are essential to ensure stable genome replication in infected epithelial cells. E6 also prevents apoptosis by interfering with Bak (Thomas & Banks, 1998), Bax (Li & Dou, 2000; Vogt et al, 2006), Fas (Filippova et al, 2004) and c-myc (Gross-Mesilaty et al, 1998), all of which are pro-apoptotic, and it also stimulates the anti-apoptotic NF-κB pathway (James et al, 2006). As well as supporting cellular survival and proliferation, E6 increases telomerase activity, which is absent from somatic cells, and thus prevents chromosome end ligation and mitotic failure (Klingelhutz et al, 1996; Plug-DeMaggio et al, 2004). The relevance of this function for the viral life cycle remains to be determined (Park & Androphy, 2002).

In addition to E6 and E7, E5 also has proliferation stimulating activity, and can cooperate with E7 to induce the proliferation of human keratinocytes (Bouvard et al, 1994a). Although E5 is the major transforming protein in BPV-1, in HPV-16 it has only a subtle role in the productive stages of papillomavirus infection. Engineered viruses mutated in E5 expression undergo reduced DNA synthesis in the suprabasal cells, but viral genome amplification, late gene expression and virion production are unaffected (Genther et al, 2003). E5 is a hydrophobic membrane protein that localises to various cellular organelles including the Golgi apparatus and the endoplasmic reticulum. It functions primarily by stimulating Epidermal Growth Factor Receptor (EGFR) activity which results in increased MAP kinase and Protein Kinase C signalling. Epithelial cells are particularly rich in EGFR, and E5 transforming activity is reduced in cells lacking EGF receptors (Pim et al, 1992). E5 probably exerts its activity by inducing endosome alkalisation and the enhanced recycling of EGFR into the cell membrane (Straight et al, 1995). Other E5 functions also include increasing host cell resistance to apoptosis through the activation of two survival signalling molecules, PI3K and ERK1/2 MAPK (Zhang et al, 2002), and contributing to immune evasion by the down-regulation of MHC class II cell surface expression (Zhang et al, 2003).

Genome amplification

Replication of the viral genome greatly increases in the upper middle layer of the epithelium, in a region that overlaps with increased cellular proliferative capacity induced by the expression of E6 and E7 (Figure 3a). As mentioned above, E2 protein levels determine its transcriptional modulation of the early viral promoter

from which E6 and E7 transcription is controlled. At the stage of viral genome amplification, E2 (as well as E1) is expressed at increased levels and therefore the expression of E6/E7 from the early promoter is expected to be eventually reduced. The activation of the late promoter (p670 in HPV-16 which lies within the E7 coding region) takes place at this stage and leads to the activation of E1, E2, E4 and E5 expression (reviewed by Doorbar, 2005). While the expression of E1 and E2 is important for viral genome replication (see above), the roles of E4 and E5 at this stage of the viral life cycle are still poorly understood.

E4 is highly expressed at this stage and its expression marks the beginning of the late events of the virus life cycle. The E4 ORF lies within the E2 sequence and the protein is expressed from transcripts that include a region within the E1 coding sequence that has been spliced into the 5' end of the E4 mRNA (Nasseri et al, 1987). E4 is predominantly cytoplasmic and is highly insoluble due to its interaction with keratin structures (Doorbar et al, 1991). The N-terminal domain of E4 mediates its binding to keratin filaments, especially to keratin 18, a member of the type 1 intermediate filament family (Wang et al, 2004). This interaction results in collapse of the keratin network in the cell which is thought to delay epithelial terminal differentiation and therefore increase the viral replication capacity and assist viral egress (Doorbar et al, 1991). Another major activity of E4 is the induction of a G2 arrest through the sequestration of active CDK1/CyclinB1 into the cytoplasm (Davy et al, 2005). The exact advantage of this cell cycle arrest for the virus is not clearly understood. One possibility is that E4 prolongs suprabasal DNA synthesis and thus enhances viral genome amplification (Nakahara et al, 2002; Nakahara et al, 2005). Additional functionally uncertain activities of E4 include binding to a DEAD box RNA helicase (Doorbar et al, 2000)

and to the mitochondria (Raj et al, 2004). The function of E4 is important for an optimal viral life cycle, as engineered viruses with disrupted E4 expression have been shown to have greatly reduced vegetative replication and late gene expression and therefore diminishes virion production (Peh et al, 2004; Wilson et al, 2005; Wilson et al, 2007).

Virion release and vaccination

At the upper layers of the epithelia, virions are produced by the assembly of viral genomes into icosohedral capsids. This requires the viral structural proteins, L1 and L2, which constitute viral capsomeres at a ratio of 30:1 (Roden et al, 1996). L1 is the major component of the capsid which can spontaneously assemble into virus-like particles (VLPs) (Kirnbauer et al, 1993), while the L2 protein is required for efficient encapsidation of the viral genomes (Roden et al, 1996). In addition, L2 can redirect L1 into promyelocytic leukaemia protein (PML) domains which most likely serve as sites for virion assembly (Day et al, 1998). The non-structural E2 protein may also play a role at this stage by binding to viral genomes and enhancing their recruitment into the sites of assembly. This association of E2 with PML bodies is possibly mediated through its direct interaction with the viral L2 protein (Heino et al, 2000).

The interesting ability of L1 to natively assemble into VLPs has helped the development of a prophylactic vaccine against the high-risk HPV-16 and -18. The vaccine acts by inducing humoral immune response against the corresponding HPV-type infection (reveiwed by Roden & Wu, 2006). The use of a vaccine against

HPV coat protein will, however, be ineffective in treating HPV-associated cancers, as these contain integrated genomes with disrupted L1 and L2 expression. In this case targeting the continual expression of E6 and E7 is a more attractive approach (see below). Such approaches may include the use of blocking peptides (Sterlinko Grm & Banks, 2004), RNA interference (Bagasra, 2005) and stimulating cytotoxic T lymphocytes to recognise infected cells (Sin, 2006).

HPV-induced malignancies

Only a very small fraction of high-risk HPV infections progress into high grade cervical neoplasia. This occurs especially in cases where viral infection is persistent and fails to be resolved by the immune system of the infected individual. In such cases, productive infection of the virus is supported only very poorly (Doorbar, 2006; Middleton et al, 2003). The normal differentiation of host cells into the upper cornified layers is essential for virion production and release. Therefore, disrupting the normal epithelial cell differentiation into the upper layers is very disadvantageous for the virus. Avoiding such a catastrophic event in the papillomavirus life cycle requires fine regulation of its gene expression as well as the activity of the expressed gene products. Upregulation of both E6 and E7 expression is generally seen in HPV-induced malignancies and viral DNA is usually found integrated into its host genome (Peitsaro et al, 2002). Integration often takes place in common fragile sites within the E2 sequence (Thorland et al, 2003) resulting in the loss of E1, E2, E4, and E5 sequences and the upregulation of both E6 and E7 expression (Corden et al, 1999). There is no evidence that integration can be followed by a recombination event which restores viral

episomes. The integration process by itself may result in genetic abnormalities of the host genome(Popescu & DiPaolo, 1990), but it is the continual expression of E6 and E7 that has been extensively studied in relation to cancer progression. Both proteins can cooperate to induce immortalization of keratinocytes (Pei et al, 1994), with the expression of E7 alone sufficient to induce DNA synthesis in differentiated keratinocytes (Cheng et al, 1995) and invasive cervical cancer in transgenic mice (Riley et al, 2003).

Although the expression of E6 and E7 is essential for a productive viral life cycle, their uncontrolled expression can be deleterious for the virus. The induction of an uncontrolled cell division state by E6 and E7 in the upper epithelial layers results in a failure of cell differentiation and virion release (Figure 3b), even in the presence of intact viral episomes that continually express E1 and E2 (Middleton et al, 2003). One way of controlling the expression of E6 and E7 is through transcriptional regulation by E2. In HPV-transformed cell lines containing integrated sequences of E6 and E7 and an intact LCR, E2 can strongly suppress transcription of both genes, resulting in cell growth inhibition (Dowhanick et al, 1995; Francis et al, 2000; Goodwin & DiMaio, 2000). Therefore, the loss of E2 (and E1) expression not only results in loss of viral replicative capacity but also in loss of transcriptional control of the viral oncoproteins. In addition, cell growth inhibition by E2 can also occur in an E6/E7 gene-repression independent pathway (Demeret et al, 2003; Webster et al, 2000). Therefore, the dramatic change in gene expression profiles that are induced by E2 (Thierry et al, 2004; Wells et al, 2003) may occur in an alternative pathway to the E2-LCR binding. These may involve the binding of E2 to various components of the cellular transcription machinery (Hadaschik et al, 2003; Rehtanz et al, 2004), or the presence of unidentified E2

DNA binding sites in the promoter region of cellular genes that could allow E2 to influence cellular gene expression.

The difference between the potential capacity of low- and high-risk HPV oncoproteins to induce cancer progression may be correlated with the sequence of events during their respective life cycles (Doorbar, 2005). High-risk HPVs replicate their genomes in the upper epithelial layers and therefore viral gene products are important to stimulate the availability of cellular enzymes required for replication. Meanwhile the productive replication of low-risk HPV occurs at lower epithelial layers where the virus can utilise the more readily available cellular replication machinery. This is apparent in the difference in activity of E6 and E7 encoded by high- or low-risk viruses. For example, the binding and inactivation of p53 and pRB by high-risk HPV E6 and E7, respectively, occurs at a much higher efficiency than that seen with the low-risk E6 and E7 proteins. In addition, using *in vitro* biological assays, low-risk HPV E6 and E7 exhibit very poor activities in comparison with their high-risk counterparts in inducing anchorage-independent growth in rodent fibroblasts and in cellular immortalisation of human keratinocytes (Barbosa et al, 1991).

The oncogenic activities of high-risk E6 and E7 will be discussed below. It is worth noting that cancer development generally involves the deregulation of multiple cellular pathways and the disruption of only a single gene product in each pathway (Vogelstein & Kinzler, 2004). HPV E6 and E7 can regulate multiple cellular pathways. Each oncoprotein, however, can result in the disruption of multiple gene products within a single pathway. Therefore, the activities of the high-risk HPV

oncoproteins, to be discussed below, may occur only following certain post-translational modifications of the viral proteins or under certain cellular conditions.

E6 transforming capacity

Although E7 is the more potent oncoprotein of high-risk HPV, the expression of E6 strongly enhances E7-induced cervical carcinogenesis in transgenic mouse models (Riley et al, 2003). Furthermore, the expression of E6 alone in the skin of transgenic mouse is sufficient for the development of carcinoma and its activities can result in the progression of benign E7-induced tumours into malignancies (Song et al, 1999).

E6 has multiple cellular binding partners and shares functional and sequence homology with a number of oncoproteins of other viruses including Adenovirus E4ORF6 (reviewed by Mantovani & Banks, 2001). The E6 ORF contains a splice donor which alternatively splices into a number of downstream splice acceptor sites, thus producing a number of shorter forms of E6 denoted E6* I-IV (Doorbar et al, 1990; Schneider-Gadicke & Schwarz, 1986). These shorter forms of E6 have been implicated in regulating the functions of the full-length protein (Pim et al, 1997).

The inactivation of the p53 tumour suppressor is a phenomenon shared among all DNA tumour viruses, and in high-risk HPV infection it is mediated by the activities of E6. Whereas many viral oncoproteins, such as Adenovirus E1B-55K and Hepatitis B virus X proteins, inactivate p53 by sequestering it into inactive complexes, E6 functions by mediating proteasome degradation of p53. High-risk

HPV E6 contains multiple contact sites that mediate the binding to p53 (Pim et al, 1994). In contrast, low-risk HPV E6 proteins bind and degrade p53 only weakly (Slebos et al, 1995; Storey et al, 1998). This also correlates with their low affinity binding to E6-AP, the ubiquitin ligase that mediates p53 degradation by the high-risk E6 proteins (Scheffner et al, 1993). Loss of p53-mediated tumour suppression leads to early tumour development (Donehower et al, 1992) and certainly contributes to E6's enhancement of E7-induced tumorigenesis (Song et al, 2000). p53 is a major transcriptional activator of cell cycle regulatory proteins and its prolonged inactivation causes dramatic changes in the cell, including alterations in the transcription of a number of genes (Kelley et al, 2005). Cells expressing E6 have increased levels of PIK1 which acts as a downstream signal from p53 to prevent cell cycle arrest and can induce chromosome mis-segregation (Incassati et al, 2006), which might contribute to the malignant phenotype as a result of E6 expression. p53 inactivation by E6 is essential for supporting E6/E7 mediated immortalisation and for the inhibition of apoptosis (Horner et al, 2004; McMurray & McCance, 2004). In these assays, telomerase activation by E6 was shown to be dispensable, although increased telomerase activity can contribute to the induction of malignant transformation and prevent cell death (Chung et al, 2005). However, additional mutants of E6 that still retain their ability to disrupt p53 activities are unable to induce cellular transformation, thus indicating the requirement of additional E6 activities that mediate malignant progression (Liu et al, 1999; Nakagawa et al, 1995; Pim et al, 1994).

In addition to p53 inactivation, E6 interacts with p300 and CBP which are transcriptional co-activators of key cell cycle regulatory proteins (Patel et al, 1999; Zimmermann et al, 1999). In the case of Adenovirus infection, targeting of

p300/CBP by E1a is essential for its transforming activities (Turnell & Mymryk, 2006). p300 and CBP can acetylate histones, allowing the activation of gene transcription, and transcription factors, such as NF-κB, p53 and c-myc, modulating their activities (Iyer et al, 2004). The exact role of p300/CBP inactivation mediated by E6 is unclear. However, E6 can complement the transformation activity of a p300-binding defective E1a mutant, suggesting that p300 binding by E6 might contribute to its transformation activities (Bernat et al, 2002). E6 can additionally regulate cellular transcription by binding and inactivating transcriptional effectors such as NF-κB and c-myc, which enhance escape from the immune response and avoid activation of apoptosis, respectively (Filippova et al, 2002; Gross-Mesilaty et al, 1998).

Additional cellular protein interactions of E6 are mediated through the extreme C-terminus sequences which are highly conserved among all high-risk HPV E6 proteins. Deletion of this domain disrupts the ability of E6 to promote skin carcinogenesis in transgenic mouse models (Simonson et al, 2005). This conserved region of E6 contains a PDZ (PSD95, Dlg and ZO-1) binding motif (XT/SXV). PDZ-containing proteins are a family of multifunctional proteins in which the PDZ domains confer protein-protein interaction capacity. E6 can interact with some members of this family, including Discs Large (Dlg) (Gardiol et al, 1999), Scribble (Scrib) (Nakagawa & Huibregtse, 2000), MAGI (Glaunsinger et al, 2000), MUPP1 (Lee et al, 2000), PTPN3 (Jing et al, 2007) and paxillin (Tong & Howley, 1997), all of which are involved in regulating various aspects of cell-cell adhesion and polarity. Several studies have shown that E6 can mediate the degradation of a number of these PDZ-containing proteins through the proteasome by recruiting the E6-AP ubiquitin ligase (Grm & Banks, 2004; Handa et al, 2007; Kuballa et al,

2007; Matsumoto et al, 2006). The advantage of this activity of E6 in the context of the life cycle of HPV is still not clear. It appears, however, to be necessary for the early proliferation events and disrupted cell adhesion and differentiation exerted by E6 (Lee & Laimins, 2004; Nguyen et al, 2003).

E7 transforming capacity

HPV E7 is arguably the most potent oncoprotein encoded by the virus. Using *in vivo* models in mice, the expression of E7 can lead to invasive cancers of the cervix (Balsitis et al, 2003; Herber et al, 1996; Riley et al, 2003). Post-transcriptionally, E7 can be regulated by both phosphorylation and the proteasome. At present, only Casein Kinase II (CKII) has been shown to phosphorylate E7 on its N-terminal domain (Barbosa et al, 1990) which has been shown to be essential to the transformation activity of E7 (see below). An additional phosphorylation site lies in the C-terminal domain of E7 that is phosphorylated in S-phase by an unknown kinase (Massimi & Banks, 2000). In addition, variation in the phosphorylation status of E7 was shown to affect the antigen recognition of the protein, suggesting that phosphorylation of E7 may regulate its secondary conformation and protein-protein interactions (Kee et al, 1998). More is known about proteasome-mediated degradation of E7 (Reinstein et al, 2000; Wang et al, 2001), where it has been shown that E7 interacts with the Skp-Cullin-F box (SCF) ubiquitin ligase complex (Oh et al, 2004a). This interaction results in increased ubiquitination of E7 and, in cells lacking a functional SCF complex, Skp2 -/- MEF cells, E7 has an increased half-life of more than an hour compared with 30min in wild-type MEF cells. The SCF complex is involved in the degradation of many cell cycle regulated proteins

including E2F-1, p27 and cyclin E (Nakayama & Nakayama, 2005; Petroski & Deshaies, 2005). Although most substrates of the SCF ligase are phosphorylated prior to ubiquitination, phosphorylation-dependent regulation of E7 ubiquitination by the SCF complex has not yet been investigated.

The localisation of E7 is still somewhat ill-defined within the cell. Indirect immunofluorescence in HPV-positive cell lines (Oh et al, 2004a) and subcellular fractionation (Sato et al, 1989) indicate that E7 is cytoplasmic. However, the nucleus is a physiologically important site for the major activities of E7 including the disruption of the pocket protein activities. Nuclear E7 has been detected using transformation assays of rodent fibroblast cells (Smith-McCune et al, 1999), epithelial raft tissues (Middleton et al, 2003) and transient transfection assays in HPV-negative cell lines (Guccione et al, 2002; Sato et al, 1989). No obvious NLS has been identified in the E7 sequence, and the mechanism of its nuclear localisation remains unidentified.

The E7 protein can be divided into three conserved regions (Figure 5); CR1-3, based on its sequence homology to other DNA virus oncoproteins; Adenovirus E1a and SV 40 Large T antigen (Phelps et al, 1988; Phelps et al, 1989). The CR2 and CR3 regions of HPV E7 mediate most of its described cellular protein interactions, however the full-length protein is critical for its optimal activity (Patrick et al, 1994).

CR1

The CR1 constitutes the first 20 amino acids of HPV-16 E7, and this region is critical for its transforming potential (Brokaw et al, 1994). Substitution of the second residue of E7 (Pro2) and a deletion mutant Δ6-10 greatly reduces its ability to induce S-phase progression and cell transformation, although these mutants still retain their pRB binding activities (Demers et al, 1996). In addition, the Pro2 mutation of E7 disrupts its ability to upregulate the activity of cyclin A which is mainly mediated through the CR2 region (see below) (Zerfass et al, 1995). Little is known about the mechanisms that lie behind the potential activities of the CR1 domain of E7. Structural studies have shown that the N-terminal domain of E7 is highly disordered, so it might be speculated that mutations within this region would have little impact on the overall structure of the E7 protein (Ohlenschlager et al, 2006). To date, only a few proteins have been shown to bind to the CR1 region of E7, these include p300/CBP-associated factor (P/CAF) (Huang & McCance, 2002) and a newly identified protein; p600 (Huh et al, 2005). P/CAF is important for the transcriptional activation of NF-κB family members which are involved in initiating cellular immune responses. The expression of E7 suppresses P/CAF-mediated activation of the NF-κB response that might be stimulated in response to viral infection. This activity of E7 is disrupted by mutating its second residue which is also required to mediate the binding with P/CAF, although an additional binding site can be found in the CR3 domain of E7 (Avvakumov et al, 2003). Meanwhile, the binding between E7 and p600 was identified using tandem affinity purification. p600 is essential for anchorage-independent growth of both HPV-negative and HPV-positive cell lines. It possesses a possible ubiquitin ligase

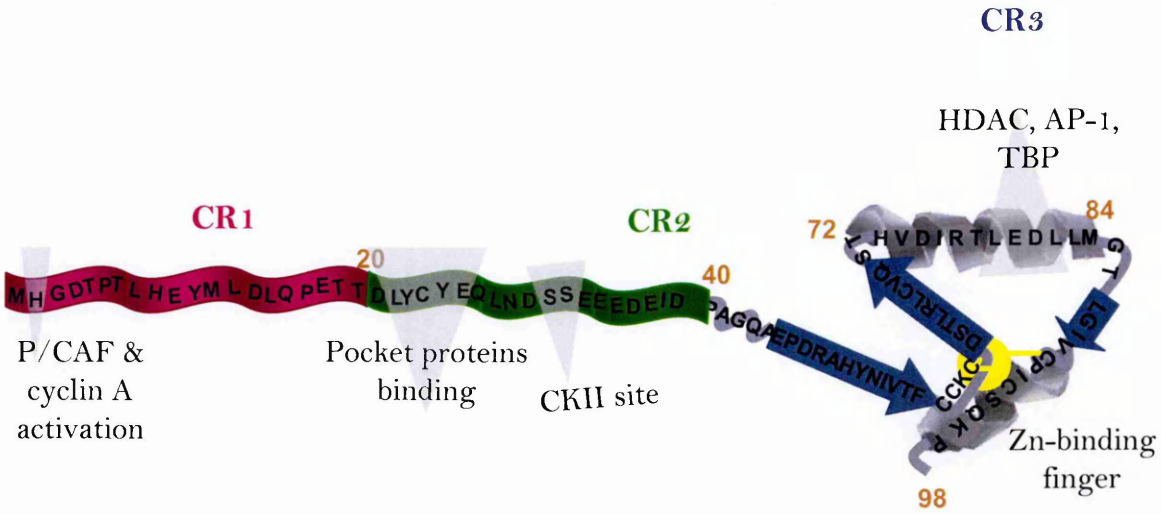


Figure 5. The E7 protein. A model of HPV-16 E7 that highlights various regions involved in its post-translational modification, binding to cellular substrates and Zn-binding domain. The amino acid residues constituting each of the conserved regions (CR) of E7 are indicated and the cysteine residues mediating the Zn-binding finger are highlighted within the CR3. Various regions that mediate binding to cellular targets of E7 are highlighted by triangular boxes. Numbers indicate the amino acid residue from the start of the E7 peptide.

domain but its role in E7's activities is still unclear. However its localisation in the cytoplasm may contribute to some of E7's cytoplasmic functions.

CR2

The CR2 region of HPV-16 E7 spans amino acids 20 to 38. It contains an LXCXE motif, which confers binding to the pocket proteins, and a CKII phosphorylation site at residues 31 and 32. The CKII consensus site has been shown to be phosphorylated by CKII *in vitro* (Firzlaff et al, 1989). Mutations in the CKII phosphorylation site in HPV-18 E7 reduce its ability to promote S-phase entry of differentiated keratinocytes cultured in rafts (Chien et al, 2000) and impair the transforming activity of HPV-16 E7 (Barbosa et al, 1990). E7 phosphorylation by CKII may affect its stability or its interaction with cellular targets. The latter has been tested in the case of E7's interaction with TBP which is enhanced by CKII phosphorylation of E7 (Massimi et al, 1996). Interestingly, the levels of cellular CKII inhibitors, MRP-8 and MRP-14, are reduced in HPV immortalised cells (Tugizov et al, 2005). Treating these cells with exogenous MRP-8/14 complexes leads to E7 hypophosphorylation and growth inhibition. Although the levels of phosphorylated E7 are reduced in these experiments, they were not completely abolished, which indicates that E7 may also contain additional phosphorylation sites. Indeed, a second phosphorylation site is located at the C-terminal region of E7, which is differentially phosphorylated during the cell cycle (Massimi & Banks, 2000).

pRB binding

The binding and inactivation of pRB by E7 has been highlighted as one of the main functions of the protein. E7 from high-risk HPV types, as well as Adenovirus E1a and SV40 LT, bind with high affinity to pRB, as well as to the other pocket protein family members p107 and p130 (Davies et al, 1993; Zhang et al, 2006). The pocket proteins bind to and inhibit E2F-mediated transcription (reviewed by Cobrinik, 2005). Their phosphorylation by cyclin dependent kinases (CDKs) inhibits their interaction with E2F, resulting in E2F transcriptional activation and cell cycle progression. Members of the pocket protein family are expressed in different tissue types and differ in their preferred targeted E2F members (Cobrinik, 2005). pRB specifically targets E2F 1-3 and is expressed in both proliferating and non-proliferating cells. p107 is predominantly expressed in proliferating cells and inhibits E2F4 activity, whilst p130 is predominantly expressed in non-proliferating cell types and inhibits E2F5 activity. The key roles of p107 and p130 in cell cycle regulation are still not clear, and attention therefore has been focused on the E7-pRB interaction. The CR2 domain of E7 mediates its binding to the B-domain of pRB, with an additional contribution from the CR3 domain of E7 (Patrick et al, 1994). E7 binds specifically to the hypophosphorylated form of pRB which is active in binding the E2F transcriptional factors (Imai et al, 1991). This binding results in disruption of pRB-E2F complexes and in destabilisation of pRB through proteasome-mediated degradation (Boyer et al, 1996; Wang et al, 2001). The disruption of pRB function by E7 results in an increased expression of cyclin A, cyclin E and CDK2 as a result of E2F-induced transcription. In addition, p14ARF expression is increased, thereby inhibiting Mdm2 activity and stabilising p53 levels (Thomas & Laimins, 1998). The stabilisation of p53 can also occur through a

p14ARF independent pathway (Seavey et al, 1999). Furthermore, E2F activation results in increased levels of p21 and p16, inhibitors of CDKs, but their negative effects on cell cycle progression is overridden by the role of the CR3 region of E7, which sequesters p21 into the cytoplasm and inhibits its activities (Westbrook et al, 2002).

pRB binding is a characteristic function of high-risk HPV E7 proteins. Mutants of E7 in the pRB binding site, however, still maintain their transforming ability (Jewers et al, 1992), meanwhile other mutants in the CR3 region (Δ79-83) which retain pRB binding, lose their immortalising phenotype (Massimi et al, 1997). Therefore, additional functions of E7 are also essential for its transformation activities (Demers et al, 1996; Jewers et al, 1992).

CR3

Whilst both the CR1 and CR2 regions of E7 are relatively unstructured and flexible in solution, the CR3 region of E7 folds into a well-structured zinc-binding domain with a *β1β2α1/β3α2* topology that is also involved in protein dimerisation (Liu et al, 2006; Ohlenschlager et al, 2006). The presence of four cysteine residues, which are highly conserved between different E7 proteins, mediate the formation of the zinc-binding finger (Figure 5). The structural integrity of this region is required for almost all of the functions of E7 and the presence of a number of charged residues mediate the binding of multiple proteins to that region (McIntyre et al, 1993). In addition to its role in binding p21 mentioned above, the CR3 region mediates the interaction with TBP (Massimi et al, 1997), TBP-associated factors

(Mazzarelli et al, 1995), members of the AP-1 family of transcription factor family (Antinore et al, 1996), the p27 inhibitor of CDK (Funk et al, 1997), M2 pyruvate kinase (Zwerschke et al, 1999), Mi2β component of the histone deacetylase (HDAC) complex (Brehm et al, 1999) and DNA methyltransferase (Burgers et al, 2007). The precise contribution of many of the CR3 interactions in the context of E7-induced transformation is not clearly understood. These interactions, however, suggest that E7 can subvert chromatin remodelling and transcriptional complexes, thus controlling cellular proliferation. By binding to, and inhibiting the activity of, HDAC complexes, E7 can increase the expression of the E2F gene by enhancing acetylation of the E2F promoter (Zhang et al, 2004). This results in increased expression of E2F-responsive genes which plays an additional role in promoting cell cycle progression. Furthermore, the binding of E7 to c-Jun, a member of the AP-1 transcription factors, results in an increased transcriptional activity of c-Jun (Antinore et al, 1996). This interaction may be important for transcriptional activation of the HPV early promoter (see above) and is also important for E7's transformation activity.

It is not clear whether the interaction between E7 and p21 occurs directly (Funk et al, 1997; Westbrook et al, 2002), although structural studies have shown that a synthetic peptide corresponding to the C-terminus of p21 can result in chemical shifts in the CR3 region of E7 (Ohlenschlager et al, 2006). The levels of p21 are elevated in the presence of E7, despite the E6-mediated repression of p53-induced expression of p21. The activity of p21, and of p27, is however inhibited in E7-expressing cells, which is essential for E7-induced cell cycle progression (Helt & Galloway, 2001; Jones et al, 1997). In the case of p21 (and possibly p27), E7 sequesters it to the cytoplasm by inducing protein kinase B (PKB) phosphorylation

of p21 and thus prevents its inhibitory activity in the nucleus (Westbrook et al, 2002). This results in an elevated CDK2/cyclin E activity which is important for G1/S-phase transition. Increases in CDK2/cyclin E levels can also be caused by direct interaction of E7 with CDK2 (He et al, 2003), by increased cyclin E expression through E2F-mediated transcription, or by up-regulated PKB expression (Pim et al, 2005) which phosphorylates and relocalises p21 into the cytoplasm (Zhou et al, 2001).

Additional functions of E7

Cell Signalling Activities

The role of E7 in regulating cell signalling is probably a reflection of some of its cytoplasmic functions (McCance, 2005). A recent interaction study has identified interaction between E7 and the Steroid Receptor Coactivator (SRC-1) (Baldwin et al, 2006). SRC-1 is an important intermediary in hormone-dependent gene expression. The expression of E7 induces the down-regulation of SRC-1-mediated transcriptional activation by retaining it in the cytoplasm and thus sequestering it from forming transcriptional regulatory complexes with P/CAF and p300 (Baldwin et al, 2006). Although p300 and P/CAF have been shown to interact with E7 (Avvakumov et al, 2003; Bernat et al, 2003), they are not involved in the interaction between E7 and SRC-1. Other signalling pathways regulated by E7 include the response to insulin-like growth factor (IGF-1). IGF-binding protein-3 (IGFBP-3) is largely expressed in senescent cells and can block proliferation in

response to IGF-1. E7 can bind and destabilise IGFBP-3 therefore inhibiting its pro-apoptotic functions in response to IGF-1 (Mannhardt et al, 2000).

E7 also upregulates PKB (or Akt) which is involved in cell proliferation and survival in response to a number of stimuli (Menges et al, 2006; Pim et al, 2005). Upon its stimulation by growth factors, PKB is activated by phosphorylation and, in turn, phosphorylates a number of downstream nuclear targets such as p21, relocalising it to the cytoplasm, and the proapoptotic Bcl-2 family member BAD, resulting in its inactivation. No direct binding between E7 and PKB has been detected and it is thought that E7 stimulates PKB signalling cascades either by decreasing PKB de-phosphorylation by PP2A (Pim et al, 2005) or in a manner dependent on pRB inactivation (Menges et al, 2006).

Centrosome Amplification

Genetic abnormalities are essential hallmarks of cancer cells (Vogelstein & Kinzler, 2004). Genomic instability is a characteristic of HPV-induced malignancies as well as many other cancer types (Lengauer et al, 1998). Whether chromosome amplification is an essential cause or a consequence of such malignancies remains to be clarified. One mechanism that can result in genomic instability is the presence of abnormal centrosome numbers at the onset of mitosis. Centrosomes are the major organising centres of the microtubule network, which is important in sustaining cell morphology, intracellular transport, cellular polarity and chromosome segregation (Fukasawa, 2002). A normal centrosome cycle involves the doubling of the mother centrosome, followed by maturation, separation and orientation into

the two poles of a dividing cell (Meraldi & Nigg, 2002). Abnormal centrosome numbers result in unequal division of chromosomes in mitosis. Throughout the cell cycle multiple proteins are involved in the centrosome cycle thus linking both cycles together. These include various protein kinases such as CDK2/cyclin E/A in centriole duplication; Aurora A and Polo-like kinases (Plk) in centrosome maturation; and CDK1/cyclinB in centrosome separation and proteolytic complexes, such as the Skp, Cullin and F-box complex (SCF) (Nakayama et al, 2000). The actual substrates of these proteins and their influence on the centrosome cycle remain to be elucidated, but deregulated expression of key proteins can result in amplified centrosome numbers.

High risk E6 and E7 proteins are both associated with genomic instability (Duensing & Munger, 2002b), although the underlying mechanism is uncertain. Both E6 and E7 can separately induce mitotic abnormalities when stably expressed in cultured cell lines or in cells derived from transgenic mice (Duensing & Munger, 2002a; Patel et al, 2004; Schaeffer et al, 2004). Further dissection of the role of each protein showed that when each protein is transiently expressed, only E7 results in immediate chromosomal abnormalities (Duensing et al, 2001a; Duensing et al, 2000). This suggests that E7 has a direct effect in inducing centrosomal abnormalities, while the effects induced by E6 might be an indirect consequence of the abrogation of p53 function (Shinmura et al, 2007; Thompson et al, 1997)

Cells expressing HPV-16 E7 appear to develop abnormal centrosome numbers before progressing to malignancy (Duensing et al, 2001a). The exact mechanism by which E7 causes this and the protein domains required remain to be clarified.

Unexpectedly, the role of pRB inactivation in centrosome over-duplication (Lentini et al, 2006; Meraldi et al, 1999) has not been linked to E7's activity (Duensing & Munger, 2003). E7, therefore, seems to function by directly abrogating the cellular machinery involved in centrosome duplication instead of its being an indirect consequence of inducing cellular proliferation. In addition, E7 upregulates CDK2/cyclin E activity (see above) which is important in licensing for centrosome duplication (Hinchcliffe & Sluder, 2001). A series of studies by S. Duensing has shown that inhibitors of RNA polymerase II and CDKs could abrogate HPV-16 E7-induced centrosome abnormalities but not normal centrosome duplication (Duensing et al, 2007; Duensing et al, 2004), suggesting that one way by which E7 may induce centrosome overduplication by activating CDK activities.

Thesis Aim

The aim of this thesis is to provide a deeper insight on molecular mechanisms that control the oncogenecity of the HPV-16 E7 protein. The first part of this thesis was based on a screen identifying various interactions between virally encoded proteins. We were particularly interested in dissecting the E2-E7 interactions since a number of recent publications have identified a transcriptionally-independent regulation of E7's activities by E2. We therefore look at the interaction between E2 and E7, dissecting the molecular domains mediating this interaction as well as the effects that this interaction has on the activity of each protein. We also extended our analysis to identify cellular proteins that may influence the activities of E7 in HPV-induced malignancies, where the expression of E2 is commonly lost. In this case, we have investigated the potential role of phosphorylation which we show influences the stability of E7. Finally, we provide evidence that both E2 and E7 can interact with Mdm2, a cellular oncoprotein, which results in modulating the activities of E2 and E7 as well as Mdm2 itself.

Results

Cross-talk between HPV-16 E2 and E7

E2 and E7 bind *in vitro*

A previous screen for potential interactions between different HPV proteins identified binding between HPV-16 E2 and E7 (Sterlinko Grm, 2005). In this study we aimed to characterise this interaction further and to dissect its biological functions. First, to confirm the interaction between E2 and E7, we performed a number of GST pull-down assays using bacterially expressed GST-tagged proteins immobilised on glutathione agarose, which were incubated with *in vitro* translated radiolabelled HPV-16 E2. All binding assays were performed for 1 hr on ice, and the reactions were extensively washed with a detergent-containing buffer. As shown in Figure 6a, HPV-16 E2 bound strongly to the GST-16E1 positive control as well as to GST-16E7. No interaction was detected with GST alone. To investigate whether E7 could also pull down E2 expressed *in vivo*, GST-16E7 was incubated with an extract from U2OS cells that had been transiently transfected with a 16E2 expression plasmid. As can be seen from Figure 6b (bottom panel), GST-16E7 retained significant amounts of 16E2 from the cell extract. In addition, using a CaSKi cell extract as a source of E7 protein, significant binding was detected with a GST-16E2 fusion protein (Figure 6b, upper panel). Overall, these results provide evidence that HPV-16 E2 and E7 can bind *in vitro*.

The *in vitro* interaction between E2 and E7 shown above might be direct, or mediated through other unknown proteins present in the reticulocyte lysate or in the cell extracts. To investigate this, we performed a direct binding assay in which

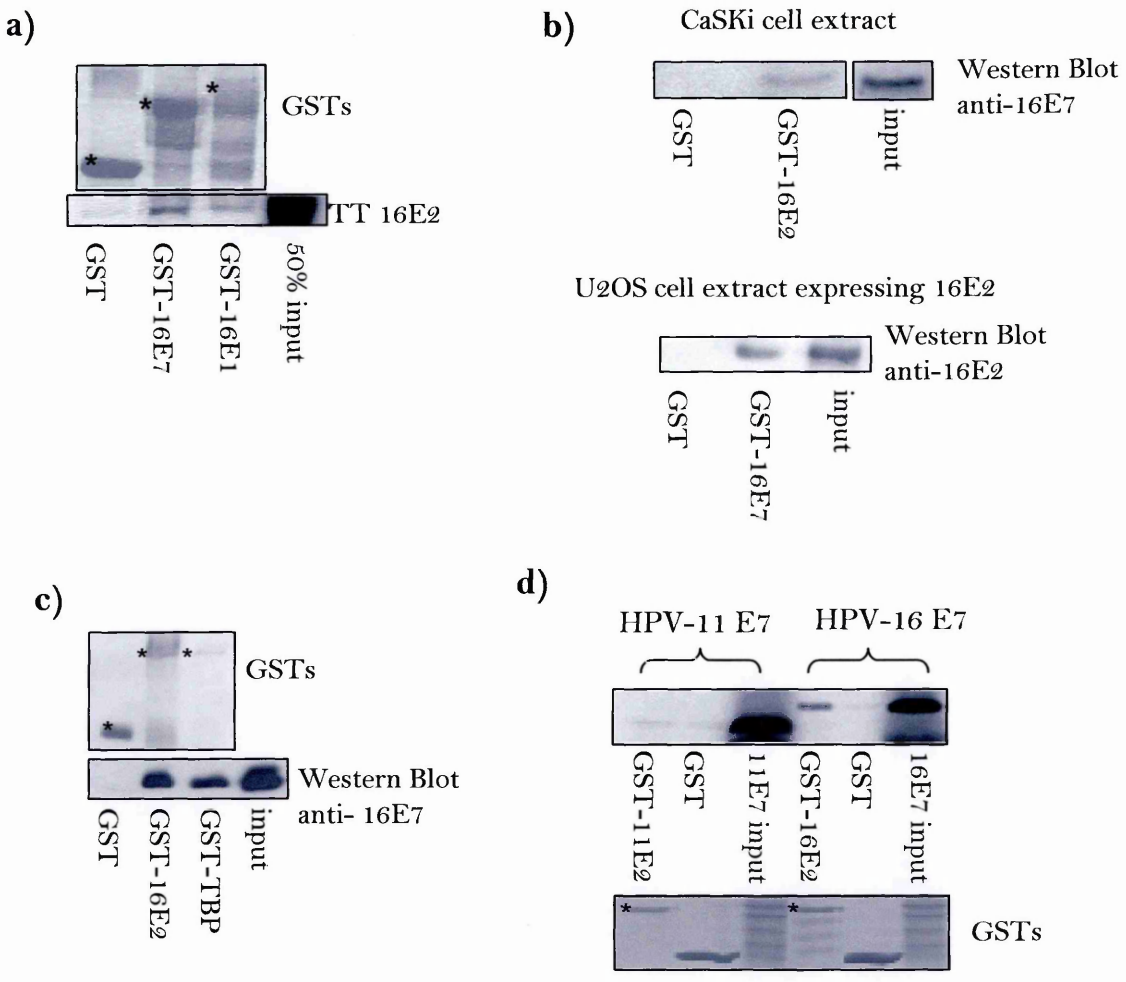


Figure 6. E2 and E7 interact *in vitro.*

a) HPV-16 E2 was *in vitro*-translated and radiolabelled in reticulocyte lysate and incubated with GST, GST-16E7 and GST-16E1. The 50% input of E2 (TT E2) is shown and bound proteins were assessed using autoradiography (bottom panel) and the input GST-fusion proteins were visualised by staining the gels using coomassie blue (upper panel). b) Extracts from U2OS cells expressing 16E2 and CaSKi cells expressing 16E7 were incubated with GST-16E7 and -16E2, respectively. Bound proteins were analysed by Western blotting using anti-E2 polyclonal (bottom panel) and anti-E7 monoclonal antibodies (upper panel). c) A direct binding assay was performed using GST-16E2 incubated with purified His-tagged 16E7. GST alone and GST-TBP were used as negative and positive controls, respectively. Bound proteins were analysed by Western blotting using anti-E7 monoclonal antibodies (bottom panel) and GST protein inputs were visualised by staining the membrane using Ponceau stain (upper panel). d) A comparison of E2 and E7 derived from different HPV types. GST-tagged HPV -11 and -16 E2 proteins were incubated with *in vitro*-translated radiolabelled HPV-11 and -16 E7 proteins respectively and bound proteins assessed by SDS-PAGE and autoradiography. 50% of the total input protein is also shown and the lower panel shows the input GST fusion proteins; (*) denotes full-length fusion proteins.

both proteins were expressed and purified from bacteria. Soluble, purified His-tagged 16E7 was incubated with purified GST-16E2 immobilised on glutathione agarose as well as with GST-TBP as a positive control and GST alone as a negative control. After 1 hr of incubation at 4°C the resin was extensively washed and the amount of E7 retained was then detected by Western blotting using an anti-E7 monoclonal antibody. The results, shown in Figure 6c, demonstrate that E7 binds E2 directly and that this interaction is comparable to the interaction between E7 and TBP. No interaction was seen between E7 and GST alone.

Having found that HPV-16E2 and HPV-16E7 could interact directly, we then wanted to assess whether this ability was conserved between low- and high- risk HPV types. To do this we performed binding assays between the respective E2 and E7 proteins derived from HPV-11 and HPV-16. As can be seen in Figure 6d, HPV-16 E7 binds to HPV-16 E2 with significantly higher efficiency than that seen between HPV-11 E2 and E7, suggesting that the E2/E7 interaction is stronger for the high-risk HPV type.

Mapping the site of interaction between HPV-16 E2 and E7

We then proceeded to map the sites of interaction between HPV-16 E2 and E7. The N- and C- terminal halves of E2 (as indicated in Figure 7a) were first *in vitro*-translated and incubated with GST-16E7 bound to agarose. After extensive washing, the bound proteins were analysed by SDS-PAGE and autoradiography. Figure 7b shows that E7 binds preferentially to the C-terminal half of E2. To further map the site of interaction, a series of truncations along the C-terminal region of E2 were expressed as GST fusion proteins, these were purified and tested

for their ability to bind *in vitro*-translated E7. As can be seen from Figure 7c, E7 only interacts with those E2 proteins that retain an intact hinge region, thereby mapping the site of E7 interaction to a region spanning amino acid residues 202-249 of E2, although a role for additional amino acid residues extending to position 306 cannot be excluded.

Then we performed a similar mutational analysis to map the site of interaction of E2 on the E7 protein. As can be seen from Figure 7d, *in vitro*-translated E2 binds preferentially to the GST-tagged C-terminal half of E7. Using a series of deletion mutants of E7 (Massimi et al, 1997) translated *in vitro*, it can be seen that the E7 mutant lacking residues 79-83 (Δ4) is defective in its ability to bind GST-tagged E2 (Figure 7e). In contrast, the other three mutants within the zinc-binding domain of E7 still retain the ability to bind E2.

HPV-16 E2 and E7 interact *in vivo*

Having shown that E2 and E7 can interact *in vitro*, we then proceeded to investigate whether we could detect the interaction *in vivo*. U2OS cells were co-transfected with HA-tagged 16E7 and un-tagged 16E2, and after 24 hrs cell extracts were incubated with anti-HA antibody cross-linked to agarose beads (Sigma). After extensive washing, the precipitated proteins were analysed by SDS-PAGE and Western blotting using a polyclonal antibody against 16E2 (Massimi et al, 1999). As shown in Figure 8, E2 co-immunoprecipitates with E7, but it is not precipitated by the anti-HA antibody if E7 is absent. These results demonstrate that E2 and E7 can form a complex *in vivo*.

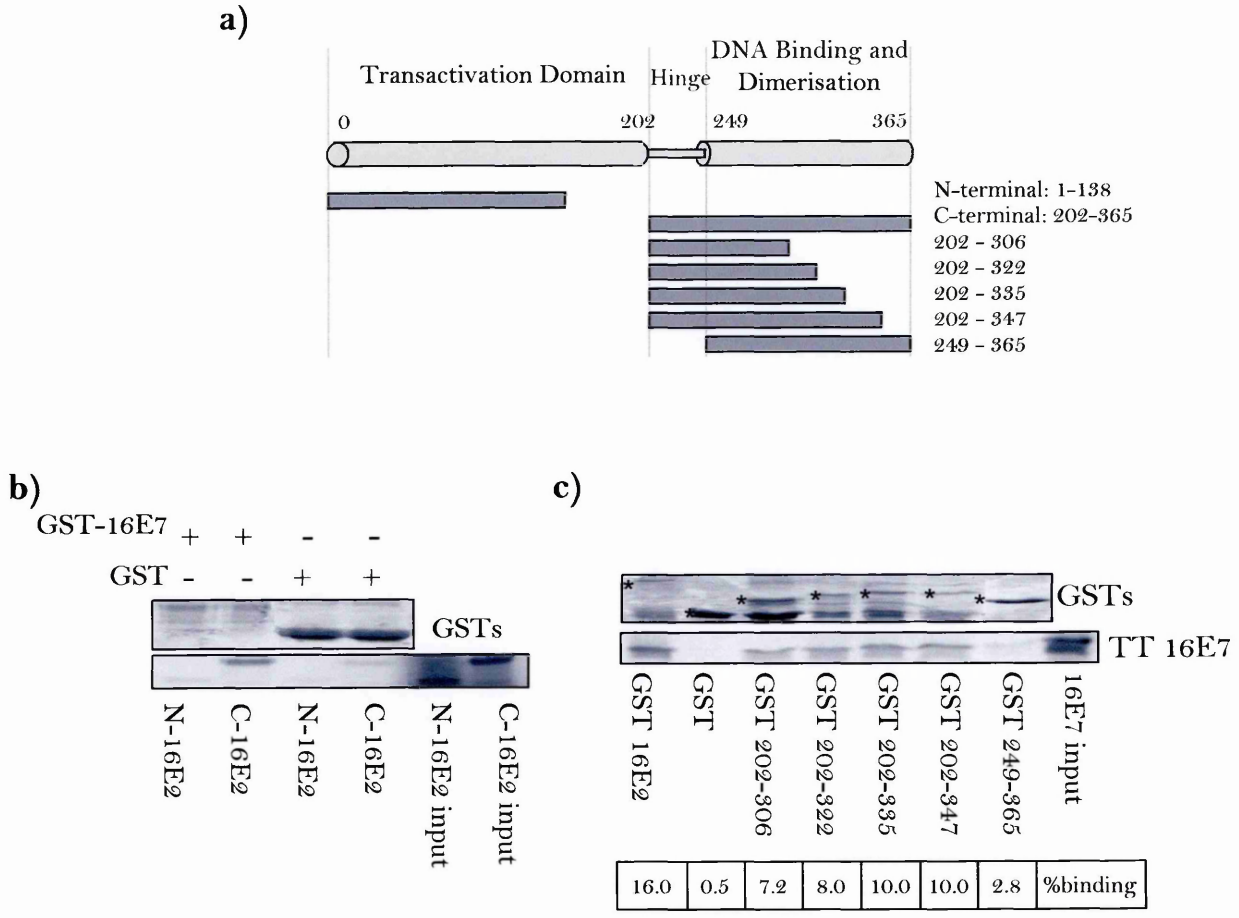


Figure 7. Mapping the sites of interaction between E2 and E7.
a) Scheme showing the E2 deletion mutants used in the study. b) GST-16E7 was incubated with the N- and C- terminal halves of radiolabelled 16E2 translated in wheat germ extract. After extensive washing, bound proteins were visualised by SDS-PAGE and autoradiography. The 50% inputs of the N- and C- terminal halves of E2 are shown (lower panel). The upper panel shows the protein stain of GST and GST-16E7. c) Truncation mutants of 16E2 were produced as GST fusion proteins, bound to resin and incubated with *in vitro*-translated 16E7 and bound proteins visualised using SDS-PAGE and autoradiography. The 50% input of E7 (TT E7) is shown (lower panel) and percentage of bound protein is also indicated. The upper panel shows the protein stain of the GST fusion proteins with (*) marking the respective full-length fusion proteins.

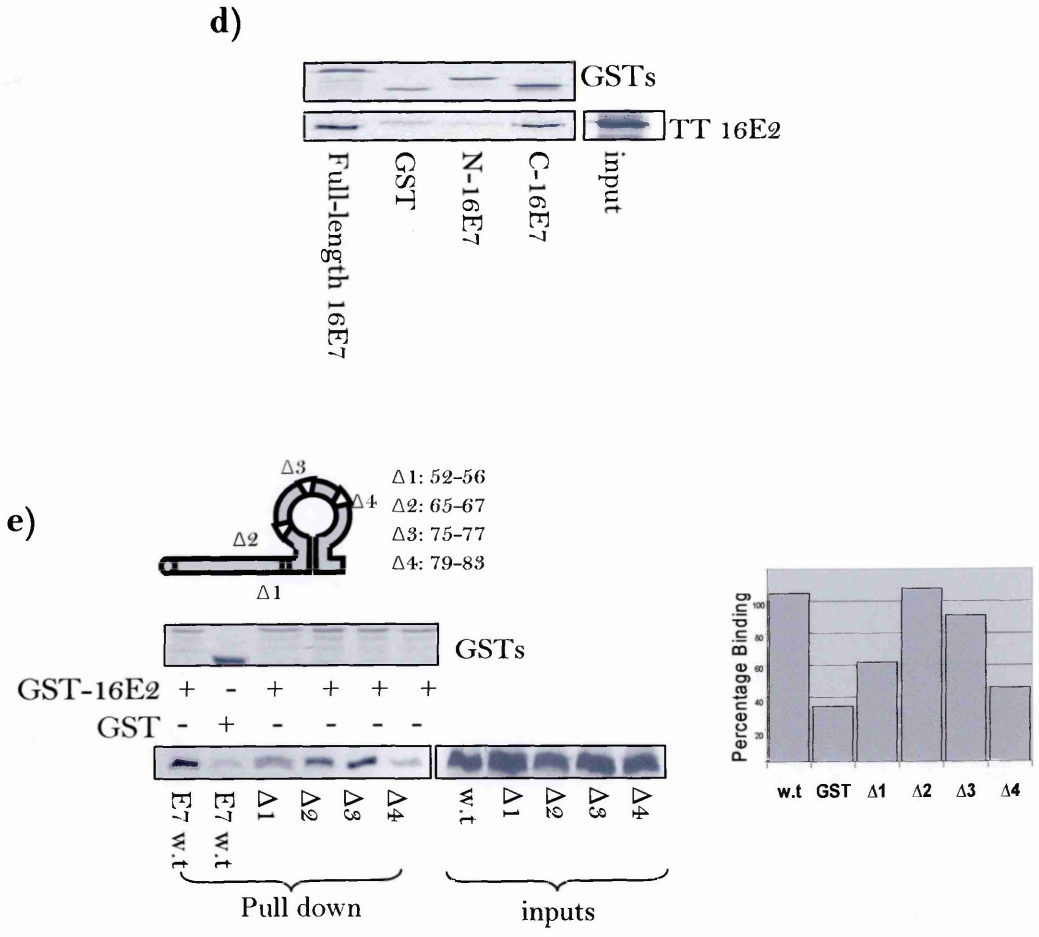


Figure 7. Mapping the sites of interaction between E2 and E7. (*cont.*)
d) The N- and C-terminal halves of 16E7 were expressed as GST fusion proteins and incubated with *in vitro*-translated 16E2. The lower panel shows the pattern of bound protein with TT E2 representing 50% of input E2 protein. The upper panel shows the protein stain of the GST fusion proteins. e) Deletion mutants of E7 (as indicated in the upper scheme) were *in vitro*-translated and incubated with GST-16E2. The protein stain of GST fusion proteins is shown in the upper gel. Bound proteins were visualised by SDS-PAGE and autoradiography (lower left gel) and 50% inputs of the deletion mutants of E7 are also included (lower right gel). The right panel shows a bar chart of percentage binding as calculated using PhosphorImager as a mean of 3 independent assays.

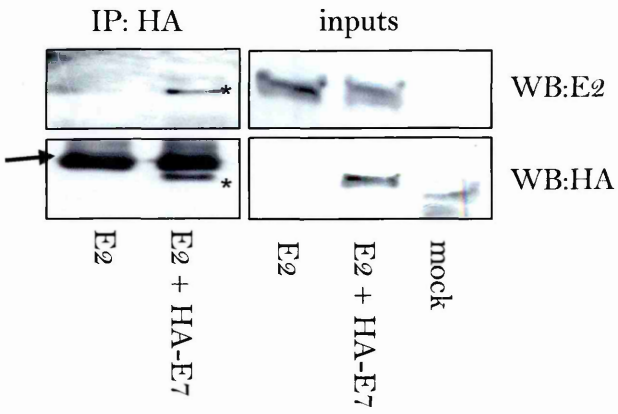


Figure 8. E2 and E7 interact *in vivo.*
U2OS cell extracts expressing 16E2 alone or with HA-16E7 were incubated with anti-HA linked to agarose beads and the precipitated proteins were detected by Western blotting using anti-E2 or anti-HA antibodies. 15% of each cell extract used for the precipitation was included as inputs. The arrow indicates IgG light chain, the (*) indicates precipitated proteins.

HPV-16 E2 increases the stability of HPV-16 E7

Upon the co-expression of E2 and E7 in U2OS cells we consistently observed that E7 levels were increased in the presence of E2, compared with expression of E7 alone. As shown in Figure 9a (lanes 2, 3 &7), when expressed alone E7 is weakly detectable in either the soluble or insoluble fractions of the cellular extract (see materials and methods), compared with the expression of β-gal used as a marker of transfection efficiency. However, in the presence of E2 the levels of E7 increase markedly (Figure 9a, lanes 4, 5, 8&9). To investigate whether this increase in E7 levels in the presence of E2 was due to the interaction between the two proteins, we repeated the assay and included two mutants of E7 within the zinc-binding finger. The results obtained are shown in Figure 9b, where it can be seen that the mutant of E7 (Δ4), that is defective for binding E2, is unaffected by the presence of E2; whilst the Δ3 mutant of E7, which retains E2 binding activity, is stabilised by E2 in a manner similar to that seen with wild-type E7.

We were then interested in investigating the effects of E2 on E7 protein expressed endogenously in CaSKi cells. Therefore, CaSKi cells were transfected with E2 and the protein levels of E7 in a total cell extract were analysed by Western blotting using antibodies against E7 and actin as a loading control. As demonstrated in Figure 10a, the protein levels of E7 are markedly increased in the presence of E2. This is independent of E7's mRNA levels as tested by semi-quantitative RT-PCR on cells transfected in a parallel experiment (Figure 10b). To test whether the increase in the levels of E7 was due to an increase in its half-life, CaSKi cells were transfected with E2 and 24 hrs later, cycloheximide was added to block protein synthesis. Cell extracts were then made at different time points and E7 expression was analysed by Western blotting. As can be seen from Figure 10c, the levels of E7

alone are significantly decreased between 15 and 30 minutes into the chase, when compared with the levels of actin which is used as a loading control of cell extracts. In contrast, in the presence of E2, the initial level of E7 is approximately two-fold higher, and this remains constant for much longer and can still be detected up to 2 hrs into the chase (Figure 10d). Similar results were also obtained using transient transfection in U2OS cells, which again was independent of any effects on E7 mRNA levels (Figure 10e). Taken together, these studies demonstrate that E2 increases the stability of the E7 protein.

E2 relocalises E7 to the insoluble fraction

In Figure 9a, E7 appears highly soluble and upon the expression of E2 it can also be detected in the insoluble fraction. This could be due to an increase in E7 levels in the presence of E2, or that E2 can specifically relocalise E7 to the insoluble fraction of the cell, which has been previously shown to include chromatin-bound E2 and core histones as markers of the chromatin (Donaldson et al, 2007; Kurg et al, 2005). To investigate this, we performed a series of assays to investigate the effects of E2 on the solubility of E7. To do this, U2OS cells were transfected in duplicates with either E2 or E7 separately, or with a combination of E2 and E7. One set of transfected cells were left untreated, while the other was pre-treated with a Triton-based pre-permeabilisation buffer prior to cell extraction. In both cases, cell extracts were separated into soluble and insoluble fractions and protein levels were detected by Western blot using specific antibodies against E2, E7 and β-gal as a marker for transfection efficiency. As shown in Figure 11a, E7 by itself is localised almost entirely within the soluble fraction of the cell and is completely

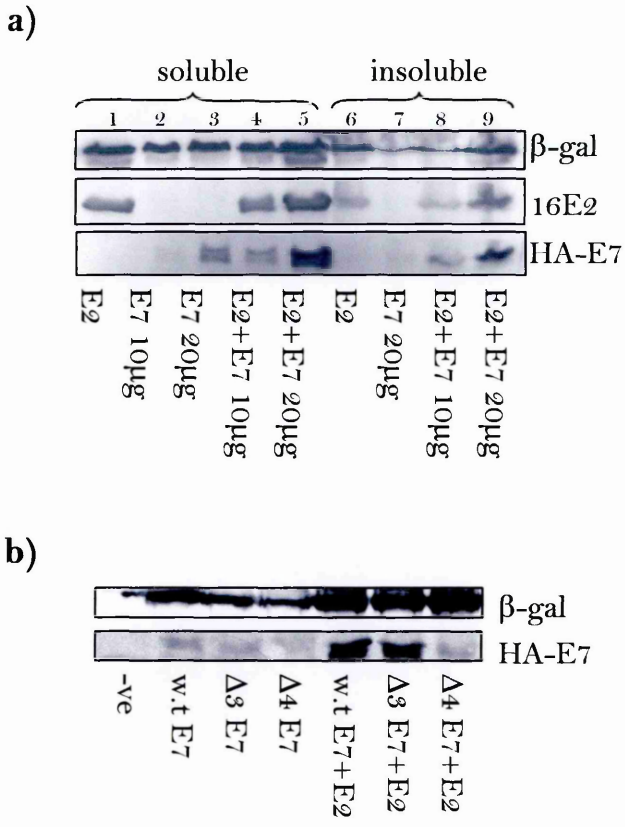


Figure 9. 16E7 protein levels are stabilised when co-expressed with 16E2 in U2OS cells.

a) Different amounts of HA-tagged E7 were transfected with or without E2. Western blots of soluble and insoluble fractions of the cellular extracts expressing different combinations of E2 and E7 were probed using polyclonal anti-E2 and monoclonal anti-HA antibodies. The expression of β-gal was used as a control of transfection efficiency. b) Cells grown in 6-well dishes were transfected with plasmids expressing wild-type (w.t) HA-tagged 16E7 and the Δ3 and Δ4 mutants of HA-16E7 with or without E2. Total cell extracts were analysed by Western blot using anti-HA or anti-β-gal antibodies.

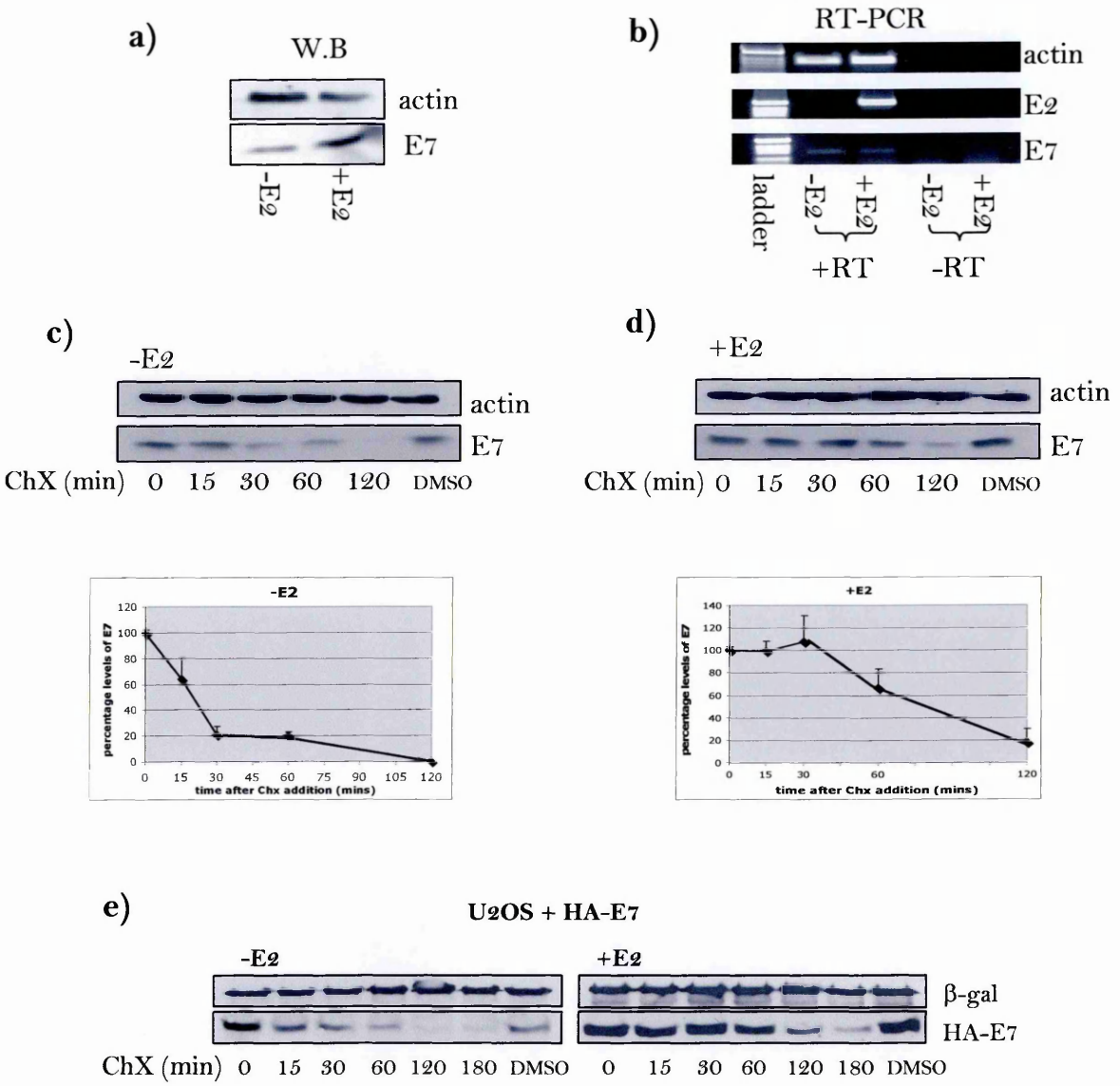
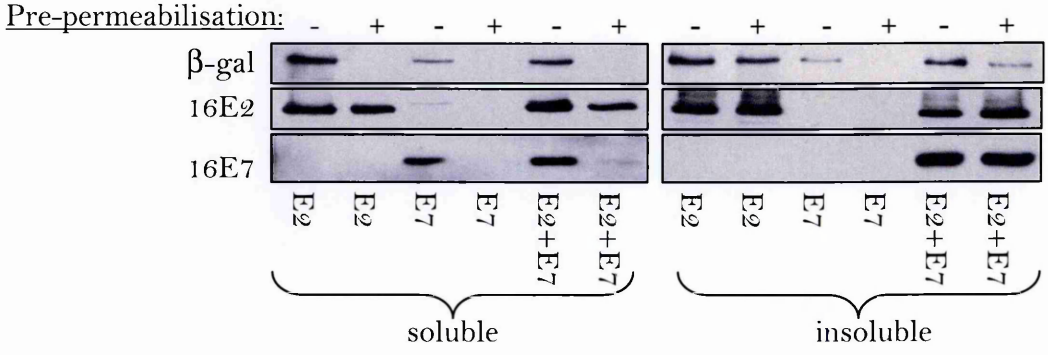


Figure 10. Increase in the stability and half-life of E7 in the presence of E2.
CaSKi cells were plated at 80000 cells per well in 6-well dishes and transfected with 3μg of empty plasmid or plasmid expressing 16E2. 24h later, the levels of E7 were analysed in total cell extracts by (a) Western blot using monoclonal anti-E7 and anti-α-actin antibodies or by (b) semi-quantitative RT-PCR using specific primers to amplify E2, E7 and actin. –RT controls were included as a control of RNA purity. c&d) CaSKi cells transfected using Lipofectamine2000 with empty plasmid (c) or plasmid expressing 16E2 (d) were treated at different times with cycloheximide (ChX) in DMSO or with DMSO alone for 120 minutes. Cells were harvested at different times (0, 15, 30, 60 and 120 minutes) after cycloheximide treatment and the protein levels of E7 were analysed by Western blot using monoclonal antibodies against 16E7 or α-actin. The intensities of the bands were measured using Adobe Photoshop and the mean results from three independent experiments are shown together with standard deviations (c&d lower panels). (e) Half life of E7 over-expressed in U2OS in the presence or absence of E2. Cells plated in 6 well dishes were transfected with 2μg of E7 alone or with 1μg of E2 expression plasmids and processed as in (c&d). β-gal was used as a transfection control and monoclonal anti-HA were used to visualise HA-tagged E7.

a)



b)

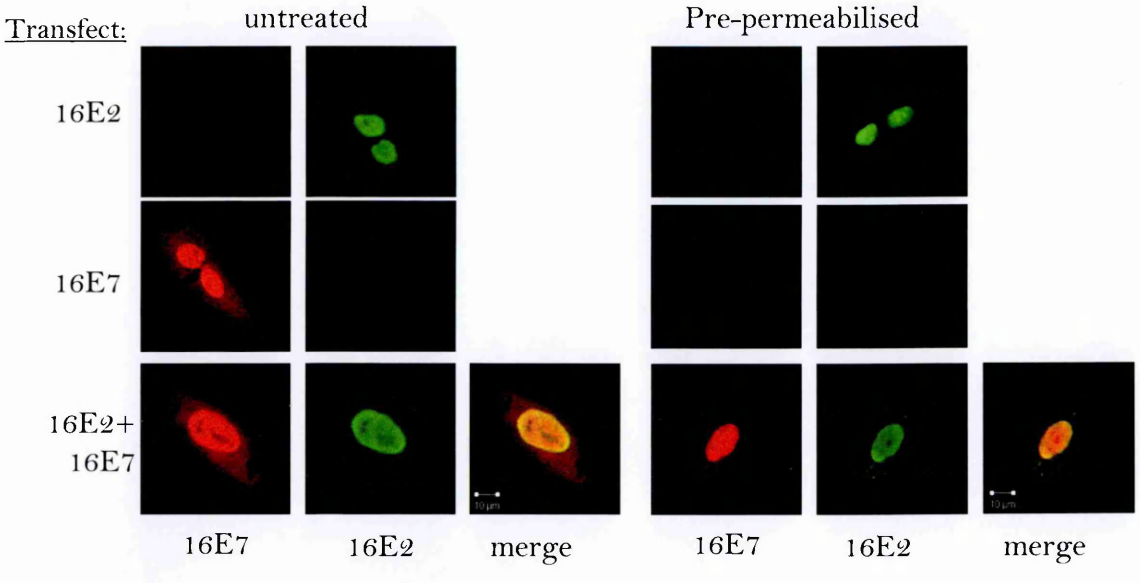


Figure 11. E7 is relocalised to the insoluble fraction of the cell in the presence of E2.

a) U2OS cells were transfected in duplicates with plasmids expressing either E2 or E7 alone or in combination along with a plasmid expressing β-gal as a control for transfection efficiency. One set of transfected cells was left untreated, while the other was treated with a pre-permeabilisation buffer for 7 minutes. In all cases, cells were then harvested and extracted with E1A extraction buffer, and the soluble and insoluble fractions of the cell were analysed by Western blotting using antibodies against E2, E7 or β-gal. b) U2OS cells grown on coverslips were transfected in a parallel experiment as above. After treatment with the pre-permeabilisation buffer, cells were fixed with PFA and analysed by immunofluorescence using rabbit anti-E2 (green) and mouse anti-E7 (red) antibodies. The scale bar shown (10 μm) is shown at the lower left side of the merged images.

lost when cells are pre-permeabilised. On the other hand, E2 appears in both the soluble and insoluble fractions and is resistant to the pre-permeabilisation treatment. In a previous work by McPhillips and colleagues the protein expression of HPV-16 E2 was lost upon treatment with extraction buffer (McPhillips et al, 2006). The difference between these results and ours could be due to the use of a different pre-extraction procedure and variation in the expression levels of E2. In those cells expressing both E2 and E7, there is an overall increase in the levels of E7 expression (consistent with results in Figure 9a). However, the bulk of this increase is in the insoluble fraction and, most importantly, the E7 protein in this fraction also becomes resistant to the pre-permeabilisation treatment. In contrast, the E7 protein that remains in the soluble fraction is still largely removed by the pre-permeabilisation treatment.

This analysis was then repeated and the pattern of E7 and E2 expression was monitored by immunofluorescence. The results obtained are shown in Figure 11b. Whilst E2 and E7 are both expressed in the nucleus, pre-permeabilisation completely abolishes E7 expression, whereas E2 can still be detected in the nucleus. In contrast, when E2 and E7 are co-expressed, there is a strong retention of E7 following the pre-permeabilisation treatment. Taken together, these results indicate that E2 can redirect E7 to an insoluble compartment within the nucleus. The possible biological consequence of this is discussed below.

HPV-16 E2 inhibits E7 induced transformation

Having shown a direct interaction between E2 and E7, we were then interested to examine whether this interaction, which results in increased stability and

relocalisation of E7, affects any of its major oncogenic activities. Previous studies have shown that the over-expression of the E2 protein resulted in cell growth arrest in cell lines containing integrated HPV DNA, due to suppression of E6 and E7 gene transcription (Dowhanick et al, 1995; Francis et al, 2000). However, we were interested in whether E2 had any direct effects upon the transformation activity of E7 in the absence of an E2-responsive promoter. To do this, we performed a transformation assay using primary Baby Rat Kidney (BRK) cells transfected with HPV-16 E7 and EJ-*Ras*, with or without HPV-16 E2 (Massimi et al, 1997). Parallel transfections using Adenovirus E1a and EJ-*Ras* were performed for comparison. After two weeks of selection the cells were fixed, stained and the number of colonies was counted. The results obtained are shown in Figure 12a, where it can be seen that HPV-16 E2 is a potent inhibitor of E7 transforming activity. This effect of E2 appears to be specific since, in contrast, E2 has no effect on the transforming activity of Adenovirus E1a (Figure 12b). To verify that the expression of E7 from the pJ4Ω plasmid was not inhibited by the E2 expression plasmid, we analysed the level of E7 gene expression 24 hrs after transfection into BRK cells by semi-quantitative RT-PCR analysis. Figure 12c shows that E2 does not inhibit E7 expression in this assay system, suggesting that E2 suppression of E7-induced transformation is at the post-transcriptional level.

Since the level of E7 protein expressed from the pJ4Ω plasmid is very low and cannot be detected by Western blotting, we sought to confirm the above observations using E7 expressed at higher levels from the pcDNA3 plasmid. Using the procedures described above for the BRK transformation assay, we optimised the expression levels of pcDNA.E7 plus EJ-*Ras* in order to achieve a comparable transforming activity to that obtained with pJ4Ω.E7 and EJ-*Ras* (Figure 12d). The

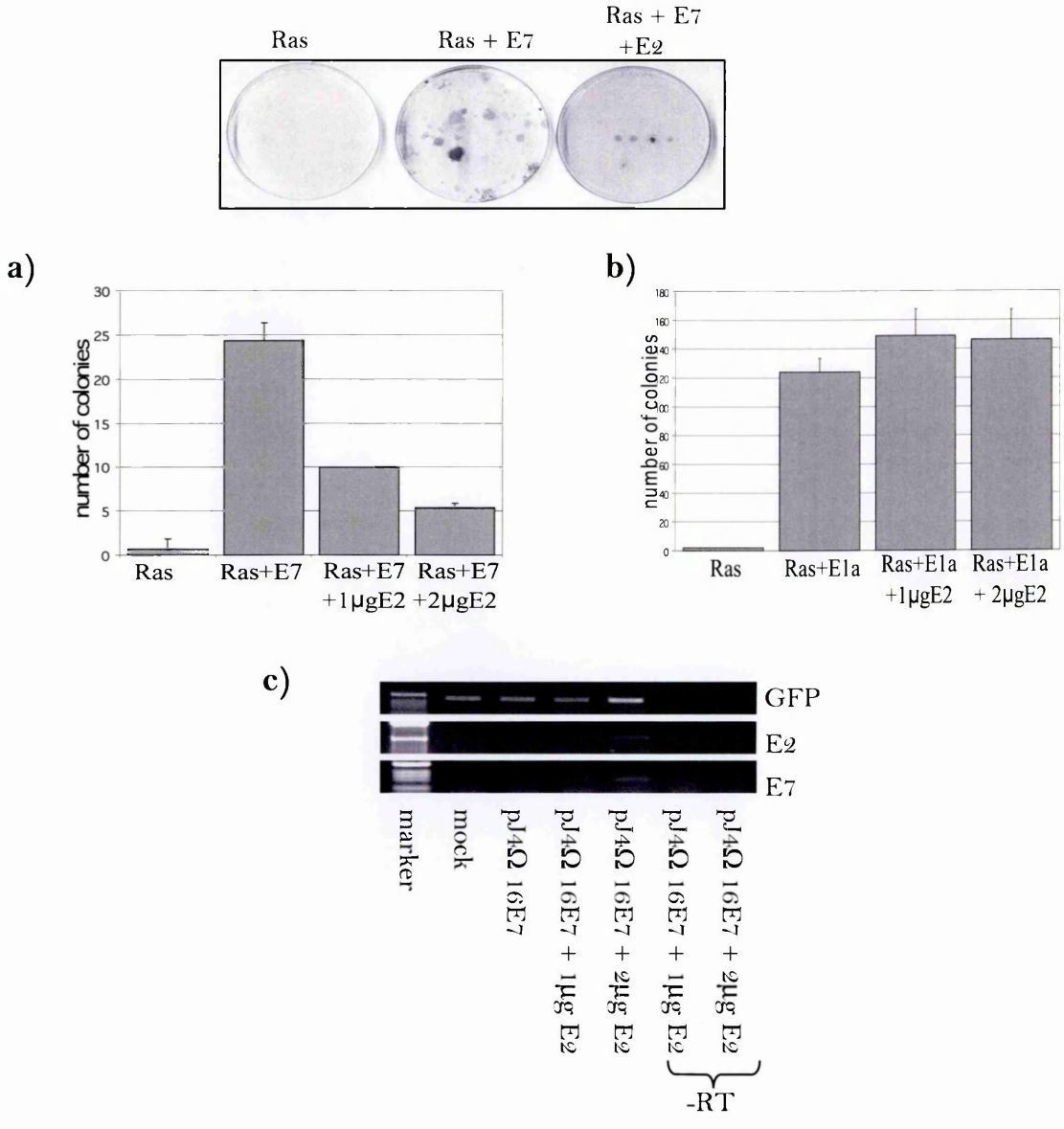
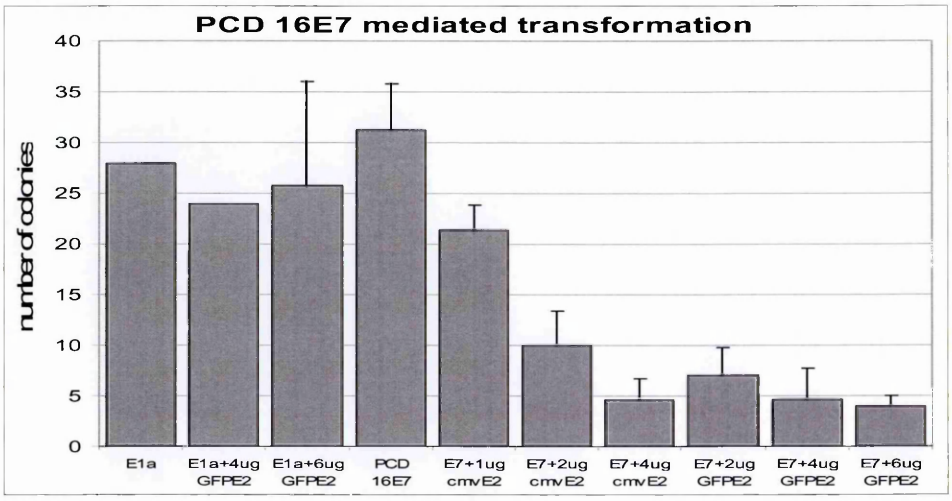


Figure 12. Transformation activity of E7 is inhibited in the presence of E2. Primary BRK cells from 9-day-old Wistar rats were transfected with 6µg of either a) pJ4Ω HPV-16 E7 or b) PCE Adenovirus E1a together with Ras as a co-operating oncogene and pcDNA carrying a selectable marker. Cells were maintained in 200µg/ml-G418 containing medium for 2 weeks, and then fixed and stained and the colony numbers were counted (as shown in the top panel). The chart shows the mean of 3 independent experiments, and error lines indicate standard deviations. c) BRK cells, treated as in (a), were transiently transfected with 6µg of pJ4Ω plasmid expressing E7 and increasing amounts of E2, along with a GFP expressing plasmid as a control for transfection efficiency. 1µg of total cell RNA was reverse transcribed and the expression of E2, E7 and GFP was analysed by semi-quantitative PCR using specific primers.

d)



e)

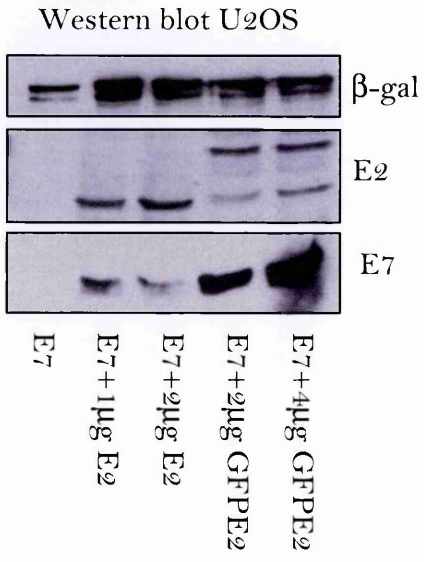


Figure 12. Transformation activity of E7 is inhibited in the presence of E2. (*cont.*)

d) BRK cells were transfected with 2μg of plasmid expressing E1a and pcDNA or 3μg of pcDNA 16E7 along with Ras expression plasmid and increasing amounts of either untagged 16E2 (cmv 16E2) or GFP-tagged 16E2 as indicated. Cells were treated and analysed as above. e) Western blot showing the levels of E7 in U2OS cells expressed from the pcDNA plasmid with various amounts of different E2 expression plasmids, as in (e). β-gal was used as a transfection control and rabbit anti-E2 or mouse anti-E7 antibodies were used to test for E2 and E7 protein levels.

expression of E2, as well as a GFP-tagged form of E2 (GFP-E2), inhibited pcDNA.E7 transformation activity. GFP-E2 had no effect on the transformation activity of E1a, confirming that E2 specifically inhibits E7's transforming activity and that this inhibition is not due solely to a general suppression of cellular transformation. Furthermore, when the levels of E7 protein were assessed by Western blotting, it was found that E2 did not inhibit E7 expression, instead it resulted in an increase in the levels of E7 (Figure 12e) which is consistent with the results presented in Figure 9a confirming an increase in the stability of E7 in the presence of E2. Overall, this set of experiments provides direct evidence that E2 inhibits the transforming activity of the E7.

Centrosome abnormalities induced by E7 are inhibited by E2

An additional cancer-associated activity of E7 has been suggested to be its ability to induce abnormal centrosome duplication and thereby contribute to the development of genomic instability (Duensing & Munger, 2002b). We therefore sought to investigate the effects of E2 upon this activity of E7. To do this, centrosome numbers were monitored in U2OS cells transiently transfected with an HA-tagged-HPV-16 E7 expression plasmid, in the presence and absence of an HPV-16 E2 expression plasmid. 24 hrs after transfection, cells were treated with nocodazole for a further 24 hrs to arrest cells in the G2/M-phase of the cell cycle where centrosome duplication would be expected have been completed. Cells were then fixed and stained for E2 and E7 using anti-E2 and anti-HA antibodies, respectively and for centrosomes using an antibody against the centrosome structural protein, γ-tubulin. The results obtained are shown in Figure 13. Cells

expressing E2 alone contained up to two centrosomes per cell, which is the characteristic number for cells arrested in G2/M (Figure 13a, upper panel). In contrast, a significant proportion of the cells expressing E7 contained elevated numbers of centrosomes, frequently possessing three or more per cell (Figure 13a, middle panel), and these results are consistent with previous publications (Duensing et al, 2004; Duensing & Munger, 2003). However, in those cells co-expressing E2 and E7, there was a reduction in the number of cells harbouring aberrant numbers of centrosomes (Figure 13a, lower panel). Figure 13b shows the collated results from multiple experiments and confirms the statistically significant effects of E2 upon the ability of E7 to induce abnormal centrosome duplication. The E7-induced increase in centrosome numbers is also observed in cells that have entered mitosis and again this is abolished by the co-expression of HPV-16 E2 (Figure 13c). These observations indicate that, while E2 does not inhibit the normal centrosome duplication cycle of the cell, its expression nonetheless inhibits E7-induced centrosome over-duplication, thus providing further evidence of E2's ability to regulate the oncogenic activities of E7 post-transcriptionally.

HPV-16 E2 inhibits E7 mediated degradation of pRB

Previous studies have suggested that the ability of E7 to induce centrosome abnormalities is independent of its ability to target pRB (Duensing & Munger, 2003). However, pRB targeting by E7 is important for its ability to cooperate with EJ-*Ras* in the transformation of primary BRK cells (Heck et al, 1992) and we were therefore interested in investigating the effects of E2 upon E7-induced degradation of pRB. To do this, pRB degradation assays were performed in SAOS-2 cells. These were transfected with a pRB expression plasmid, together with appropriate

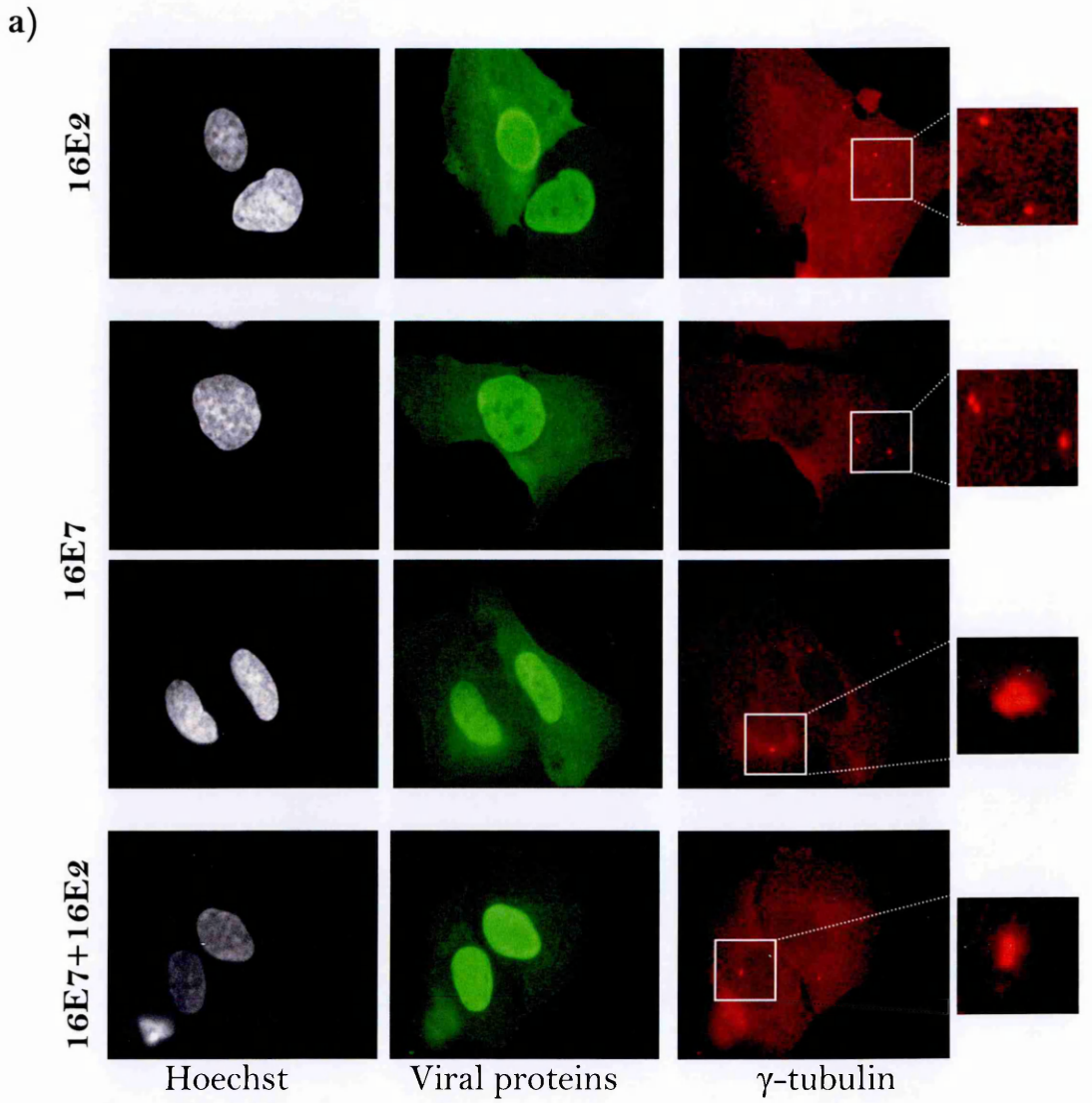
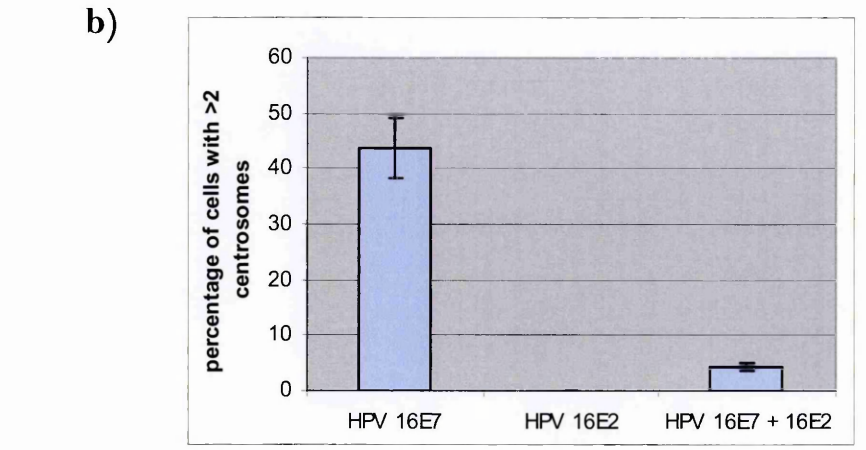
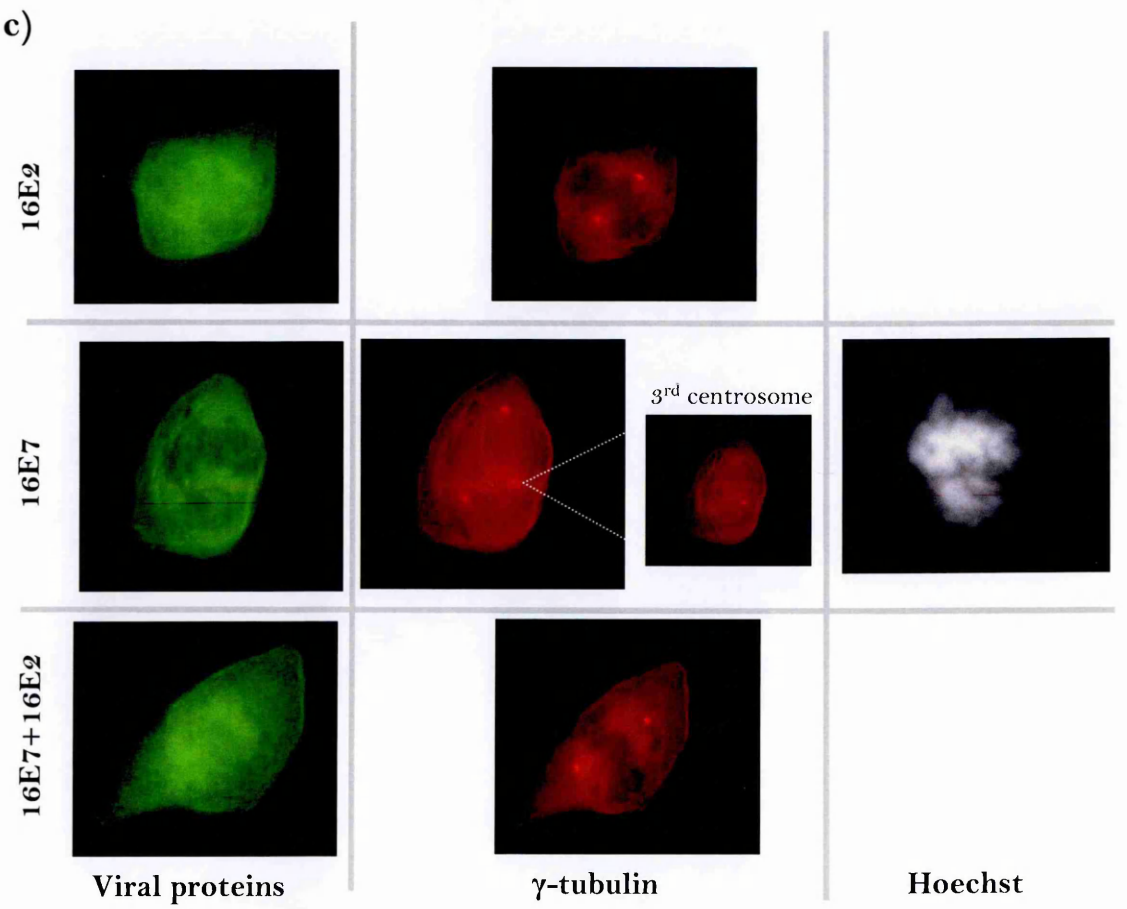


Figure 13. Effects of E2 on centrosome abnormalities induced by E7.
U2OS cells grown on coverslips were transiently transfected with HA-tagged 16E7, untagged 16E2, or both E2 and E7 expression plasmids. 24 hrs after transfection cells were treated with nocodazole for a further 24 hr period to arrest cells in G2/M phase. Then, cells were fixed with PFA and stained using Hoechst to visualise genomic DNA, polyclonal antibodies against E2 or anti-HA. Monoclonal anti-γ-tubulin was used to visualise centrosomes. a) typical centrosome numbers in cells expressing E2, E7 or E2 with E7. A magnification of the centrosomes is shown on the right. b) Statistical analysis of centrosome numbers are shown in the graph which represents the results of 3 different assays and a total of approximately 150 cells counted for each transfection along with the standard deviation obtained. c) The staining of cells that have entered mitosis processed as for panel (a).

Figure 13 (*cont.*)





combinations of untagged HPV-16 E7 and HPV-16 E2 expression plasmids. After 24 hrs, the levels of pRB protein were ascertained by Western blotting and the results obtained are shown in Figure 14. As can be seen, E7-induced degradation of pRB was rescued in the presence of E2. These results demonstrate that E2 can inhibit E7-induced degradation of pRB and provide a molecular explanation for the ability of E2 to inhibit E7's transforming activity in BRK cells.

E7 up-regulates E2-dependent transcriptional activation

Having shown that E2 binds and stabilises the E7 protein (Figures 9 and 10), but at the same time inhibits its transforming activity (Figure 12), we were next interested in investigating the potential effects of E7 upon the functions of E2. Since E7 can co-localise with chromatin-bound E2, we aimed to test whether, by doing so, E7 can affect E2-mediated transcriptional activation. Previously it was shown that E2 binds to its consensus sequence and can activate transcription from a promoter containing its DNA binding site (Donaldson et al, 2007). Here, we used a Firefly luciferase reporter construct that contains 6 synthetic tandem repeats of the E2 DNA-binding site upstream of the luciferase gene. This construct was transfected into either U2OS (p53/pRB positive) or SAOS-2 (p53/pRB negative) cells, together with GFP-tagged E2 and untagged-E7 expression plasmids, and the unrelated *Renilla* luciferase gene which is used to control transfection efficiency. 24 hrs after transfection, luciferase activity was measured and results are shown in Figure 15a. As can be seen, the transcriptional activation by E2 increases with increasing amounts of E7 in both cell lines, meanwhile Western blot analysis shows that the levels of E2 protein remain constant (Figure 15b). These results

indicate that E7 enhances E2 transcriptional activity in a manner that is independent of pRB or p53 activities.

E7 co-localises with E2 on mitotic chromosomes

A recently described chromatin-mediated function of E2 is viral genome segregation in mitosis which involves its binding to mitotic chromosomes (Skiadopoulos & McBride, 1998). This activity of E2 has been mainly studied using E2 protein derived from BPV-1, which was shown to bind to the cellular protein Brd-4 on the chromatin (You et al, 2004). Prior to investigating the potential effects of E7 upon E2 localisation during mitosis, we sought to examine whether HPV-16 E2 could bind to mitotic chromosomes in a similar manner to what has been reported for BPV E2. To do this, E2 was over-expressed in U2OS cells; 48 hrs later the cells were fixed and E2 was detected using a polyclonal antibody against HPV-16 E2. The cells were also stained with Hoechst to visualise cellular chromosomes. The cells were not synchronised by drug treatment and visualisation of the chromosomal patterns was used to determine the stage of mitosis (Scholey et al, 2003) in which the cells were fixed. As can be seen from Figure 16a, HPV-16 E2 shows a diffused nuclear staining in interphase cells, and during the initial stages of mitosis it is excluded from condensed chromosomes (Figure 16b). As the cell enters telophase, E2 localises to mitotic chromosomes (Figure 16b, lower panel).

Since both E6 and E7 are known to induce chromosomal segregation defects (Duensing et al, 2004), we were then interested to see whether they exert these effects by binding directly to the chromosomal arms. Neither E7 nor E6, when

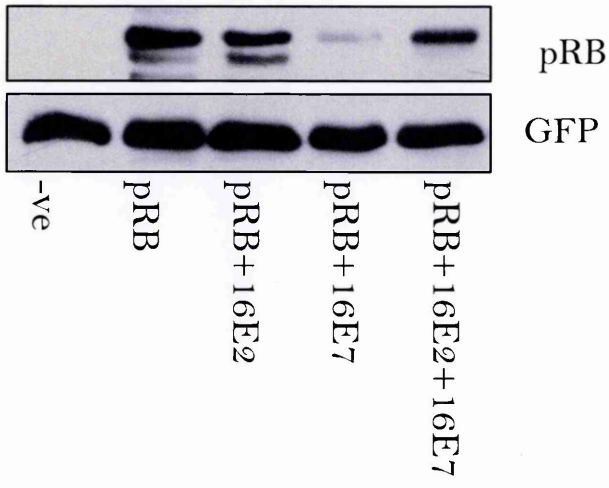
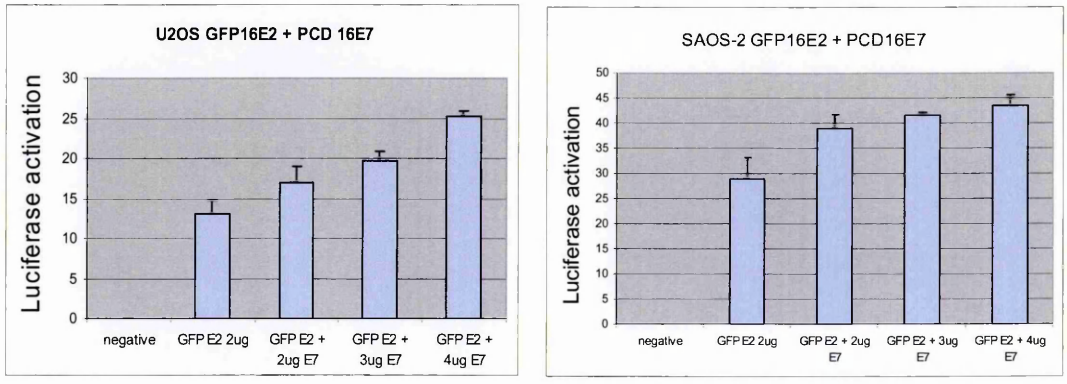


Figure 14. E2 inhibits E7-mediated degradation of pRB.
SAOS-2 cells were transfected with a pRB expression plasmid, along with either CMV.16E2 or PCDNA.16E7 alone or in combination, as well as a GFP expressing plasmid as a control for transfection efficiency. 48 hrs after transfection, cells were harvested in E1A buffer, and protein levels analysed by Western blot using antibodies against pRB and GFP.

a)



b)

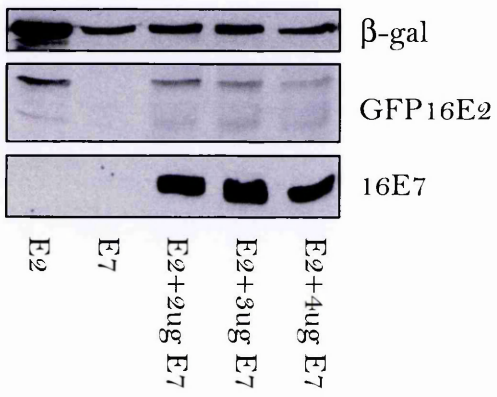


Figure 15. E7 enhances the transcriptional activity of E2.
a) U2OS and SAOS-2 cells were transfected with 2μg of GFP-tagged E2 expression plasmid (GFPE2), along with increasing amounts of pcDNA.16E7 as well as a luciferase expression plasmid containing 6X E2 binding sites and *Renilla* as a control for transfection efficiency. Graphs show the percentage activation of the luciferase gene calculated as a mean of three independent experiments. b) U2OS cells transfected as above were analysed for the levels of E2 in the presence of increasing amounts of E7 by Western blot using polyclonal anti-E2 and monoclonal anti-E7 antibodies.

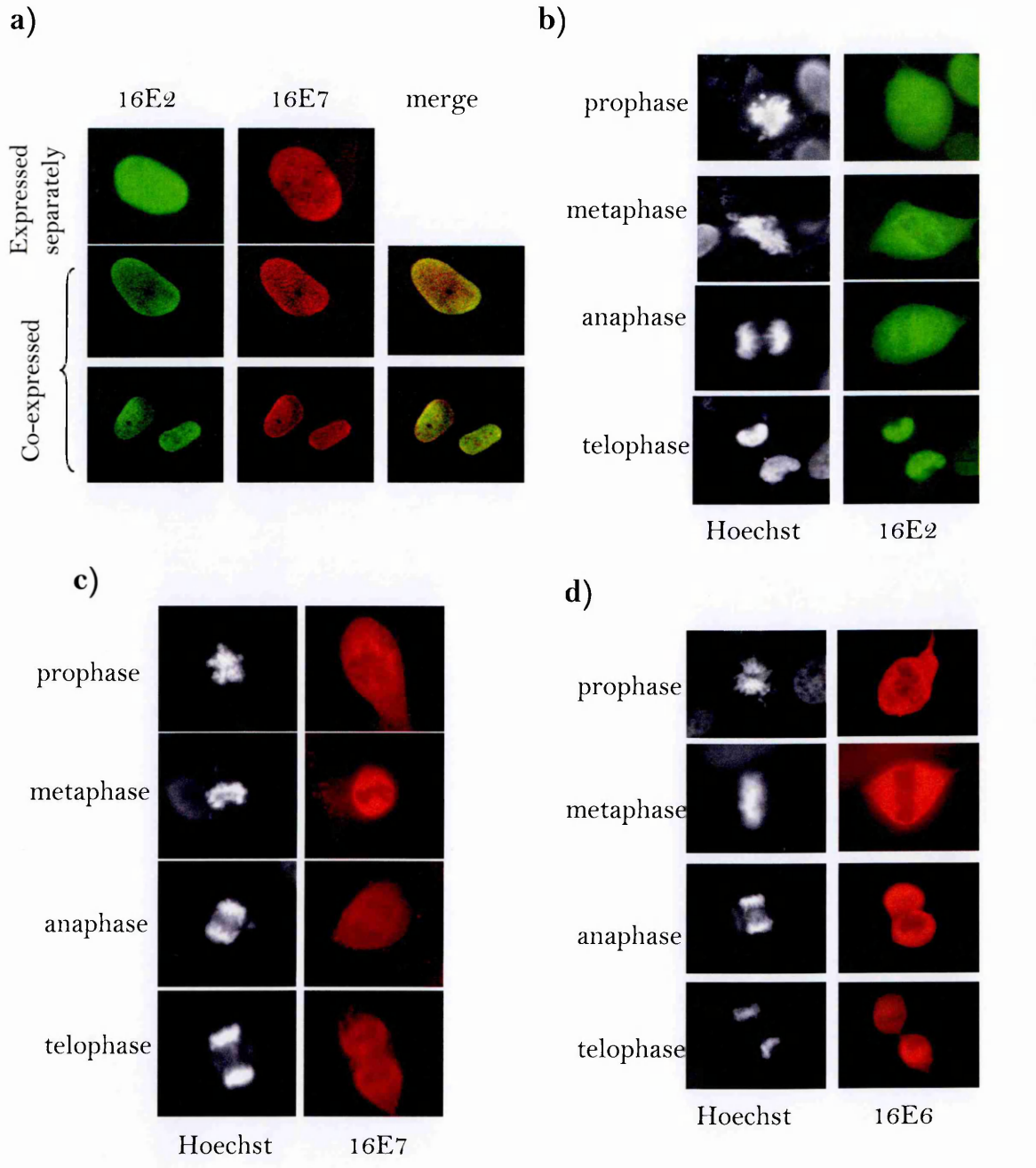


Figure 16. The localisation of E2, E7 and E6 in U2OS cells.
U2OS cells grown on cover-slips in 6-well dishes were transfected with plasmids expressing 16E2 and/or HA-tagged 16E7 and 16E6. 48 hrs after transfection, cells were fixed and probed with rabbit anti-E2 and mouse anti-HA antibodies, followed by FITC-conjugated goat anti-rabbit (green, for E2) and rhodamine-conjugated goat anti-mouse antibodies (red, for E7 and E6). The slides were scanned using Leica DMLB fluorescence microscope. a) Interphase U2OS cells were visualised for separate or co-expression of the E2 and E7 proteins. b) Localisation of 16E2, c) 16E7 and d) 16E6 at different stages of mitosis. Chromosomes are visualised using Hoechst stain.

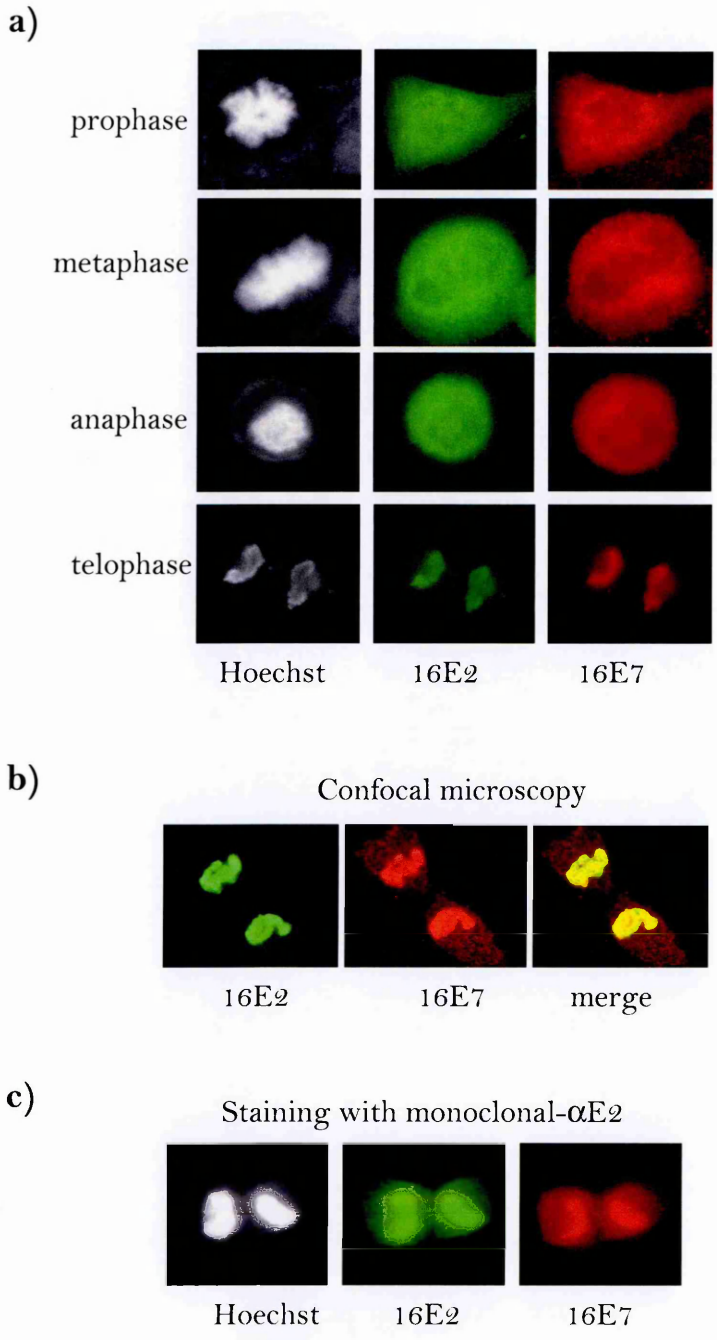


Figure 17. E2 recruits E7, but not E6, to mitotic chromosomes at telophase. U2OS cells were processed as described for Figure 7. a) The co-expression of E2 and E7 at different stages of mitosis. Telophase cells were also scanned using confocal microscopy (b) where E2 is green, E7 red and the merged image shows clear confocality. The same assay was also done using anti-E2 monoclonal antibodies (mono-αE2) together with rabbit anti-HA antibodies to detect E7 (c).

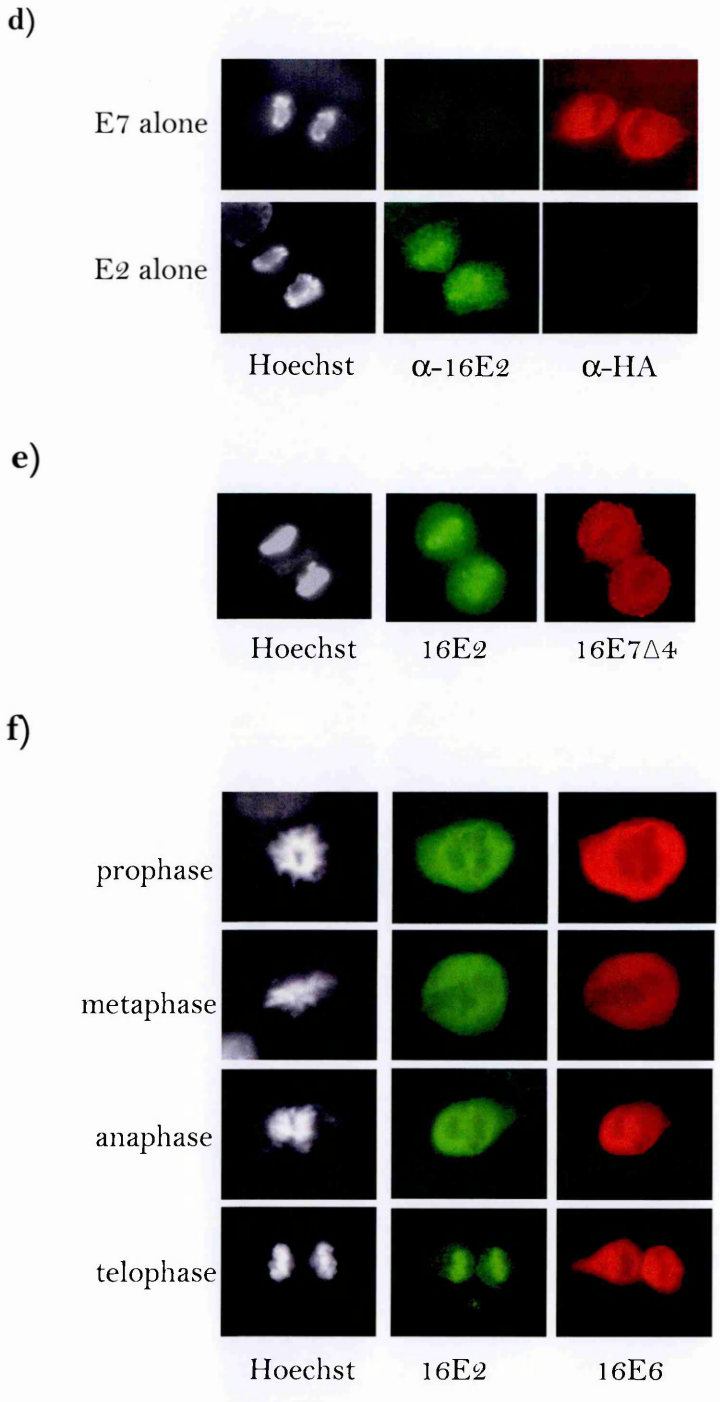


Figure 17. E2 recruits E7, but not E6, to mitotic chromosomes at telophase. (*cont.*)

d) The specificity of the antibodies used in (a; left panel) was verified by staining telophase cells expressing either HA-tagged E7 alone (upper panel) or E2 alone (lower panel) with both rabbit anti-E2 and mouse anti-HA antibodies. e) Localisation of E2 and the E7 Δ4 deletion mutant during telophase. Note chromosomal localisation of E2 but chromosomal exclusion of the E7 mutant. f) The localisation of the HA-tagged 16E6 protein co-expressed with E2 throughout mitosis showing no recruitment of E6 onto mitotic chromosomes.

transfected alone, were detected on mitotic chromosomes in any of the stages of mitosis; they show instead diffused staining and chromosomal exclusion (Figures 16 c and d). However, when HA-tagged E7 (stained with monoclonal anti-HA antibodies) is co-expressed with E2 (detected using polyclonal anti-E2 antibodies), it can clearly be detected on mitotic chromosomes together with E2 at telophase (Figure 17a, lower panel). The co-localisation between E2 and E7 was also confirmed by scanning with confocal microscopy (Figure 17b). As an additional control, E2 was also detected using a previously described monoclonal antibody (Hibma et al, 1995) together with a rabbit anti-HA antibody to detect E7 (Figure 17c) and a similar pattern of expression was observed. To further verify the specificity of the antibodies used in the fluorescence experiments, telophase cells expressing either E2 or HA-tagged E7 alone were also stained with both rabbit anti-E2 and mouse anti-HA antibodies. Figure 17d shows that in the absence of the antibody-specific protein, a very low background staining was obtained, indicating no cross-reaction between the different antibodies and proteins. To verify that the E2-induced re-localisation of E7 onto chromosomes was dependent upon the association between E2 and E7, we repeated the assay using the Δ4 mutant of E7 which cannot bind to E2. As can be seen from Figure 17e, E2 shows clear co-staining with the chromosomes whilst the E7 Δ4 mutant does not. Finally, the specificity of the re-localisation was further confirmed by the inclusion of HA-tagged 16E6, which was previously shown to interact with E2 (Grm et al, 2005), and where no E2-induced alteration of the pattern of E6 expression was seen during mitosis (Figure 17f). These results demonstrate a specific re-localisation of the E7 protein onto mitotic chromosomes as a direct result of its interaction with E2 during telophase.

Regulation of E7 by Phosphorylation

The identification of a Pin1 binding site on HPV-16 E7

Since the above data had indicated that E7 was post-transcriptionally regulated by E2, we were keen to investigate the underlying mechanism. We asked whether it might be related to phosphorylation events on E7 having direct effects on protein turnover. We therefore scanned the E7 sequence for the presence of other additional regulatory sequences; in addition to the previously described CKII phosphorylation site; we could not identify any strong matches to kinase phosphorylation sites but we could detect a potential binding site for the prolyl isomerase Pin1. Pin1 regulates the activity of numerous key cell cycle regulatory proteins such as p53, c-myc and cyclin E. It binds its targets by recognising proline residues preceded by a phosphorylated serine or threonine residue (pSer/Thr-Pro). A potential Pin1 binding motif was identified at amino acid residues 5 and 6 in the CR1 domain of HPV-16 E7. To verify the binding between E7 and Pin1, we conducted an *in vitro* binding assay in which, *in vitro* translated 16E7 was incubated with GST-Pin1. As can be seen in Figure 18a, *in vitro* translated E7 bound to GST-Pin1 but not to GST alone. Having shown an *in vitro* binding between E7 and Pin1, we then tested whether the expression level of E7 *in vivo* is affected in the presence of Pin1. To do so, HA-tagged E7 expression plasmid was co-expressed in U2OS cells together with an HA-tagged Pin1 expression plasmid. Figure 18b shows that the level of E7 is increased in the presence of Pin1. To show that the effect of Pin1 on E7 expression is dependent on the phosphorylation of E7, we additionally over-expressed the protein phosphatase 2A (PP2A) subunit which was

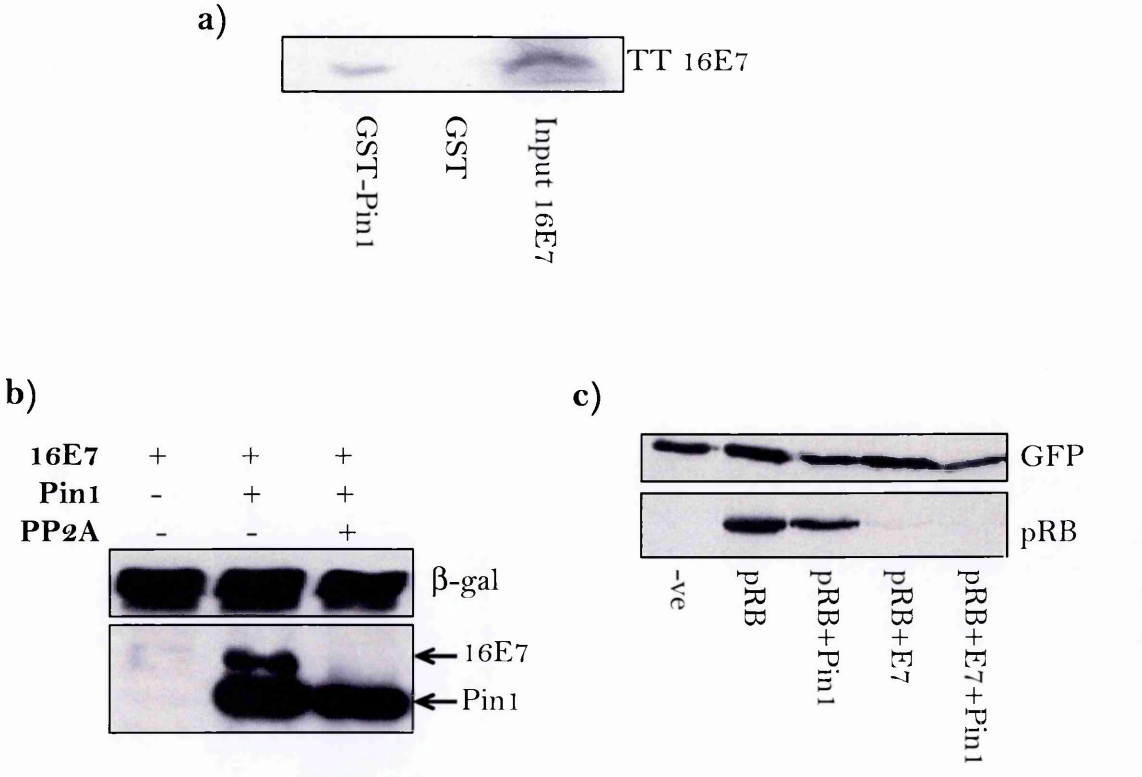


Figure 18. Pin1 binds E7 and increases its levels *in vivo*.
a) E7 and Pin1 can bind *in vitro*. GST-tagged Pin1, as well as GST-alone as a negative control, were incubated with *in vitro* translated and radiolabelled E7 for 1hr on ice. Following extensive washing with a detergent containing buffer, the amount of binding was detected using SDS-PAGE and autoradiography. b) Pin1 stabilises the levels of E7 *in vivo*. U2OS cells were transfected using HA-tagged E7, along with HA-tagged Pin1 and PP2A expression plasmids. 24 hrs after transfection, protein levels were analysed by Western blot using anti-HA antibodies and β-gal as a transfection control. c) Pin1 does not perturb pRB degradation mediated by E7. SAOS-2 cells were transfected using pRB expression plasmids, as well as pcDNA.16E7, HA-Pin1, or both E7 and Pin1 expression plasmids. Protein levels were analysed using Western blot 24 hrs after transfection using antibodies against pRB or GFP as a transfection control.

shown previously to interact with HPV-16 E7 (Pim et al, 2005) as well as with a number of other virally encoded proteins (Van Hoof & Goris, 2004). We speculated that if the activity of PP2A can result in the reduction of E7 phosphorylation then the expression of Pin1 will no longer affect E7 levels. Indeed, the co-expression of Pin1 and E7 in the presence of PP2A diminished the increase in E7's levels (Figure 18b) thus indicating that the phosphorylation of E7 is important for its stabilisation by Pin1. Having shown previously that E7 when stabilised by E2 loses some of its functional activities (Figures 12, 13 and 14), we were interested to test whether the stabilisation of E7 induced by Pin1 affects its ability to degrade pRB. Using an *in vivo* degradation assay of pRB in SAOS-2 cells, we observed that the expression of Pin1 by itself does not affect pRB levels, and when co-expressed with E7 it does not impair the E7-mediated degradation of pRB (Figure 18c). Overall, these results provide new evidence that Pin1 binds to E7 and increases E7 protein levels, however, it does not appear impede E7-mediated degradation of pRB.

Modulation of E7 levels by phosphorylation

Having shown above that Pin1, whose interaction with various proteins is determined by their phosphorylation, binds to and affects the stability of E7, we speculated whether phosphorylation by itself might play a role in regulating E7 levels. To test this, E7 was transiently expressed in the presence of a wild-type PP2A subunit, a dominant negative mutant of PP2A (PP2A-DN), and okadaic acid (OA), which is an inhibitor of endogenous PP2A activities. Figure 19a shows that the levels of E7 are reduced upon the expression of PP2A. In contrast, higher E7 levels were detected in the presence of either PP2A-DN or OA. This indicates that

inhibiting the dephosphorylation of E7, and thus increasing its phosphorylation, results in its increased levels and also suggests that phosphorylation may modulate the stability of E7. Until now, two phosphorylation sites have been identified on E7 including the CKII phosphorylation site (residues 31 and 32) (Firzlaff et al, 1989) and serine 71 which is phosphorylated by an unknown kinase (Massimi & Banks, 2000). To examine whether E7 mutants harbouring substitutions in the CKII recognition consensus (E7 31/32) or in serine 71 (E7 S71) are resistant to OA treatment, we treated U2OS cells transiently expressing E7 31/32 or E7 S71 with OA as before, and monitored protein levels using Western blotting. As can be seen from Figure 19b, both mutants of E7 are stabilised in the presence of OA in a similar fashion to the wild-type E7 protein. This suggests that additional phosphorylation sites on E7 are important for controlling its stability.

We then sought to identify potential kinases that would alter the stability of E7. Since phosphorylation plays a potential role in regulating E7 levels, we reasoned that inhibiting the activity of a stabilising kinase might lead to a reduction in E7 levels. To test this, cells expressing E7 were treated with a number of kinase inhibitors and the changes in E7 levels were assessed using Western blotting. The various inhibitors used (Table 3) include the cell cycle inhibitors, aphidicolin and nocodazole, plus inhibitors of Aurora A kinase and PIK3: none of these greatly affected the expression levels of E7 (Figure 19c). In contrast, treating cells with the CDK inhibitor, roscovitine, drastically reduced the expression levels of E7 suggesting that the activity of CDK can influence the stability of E7.

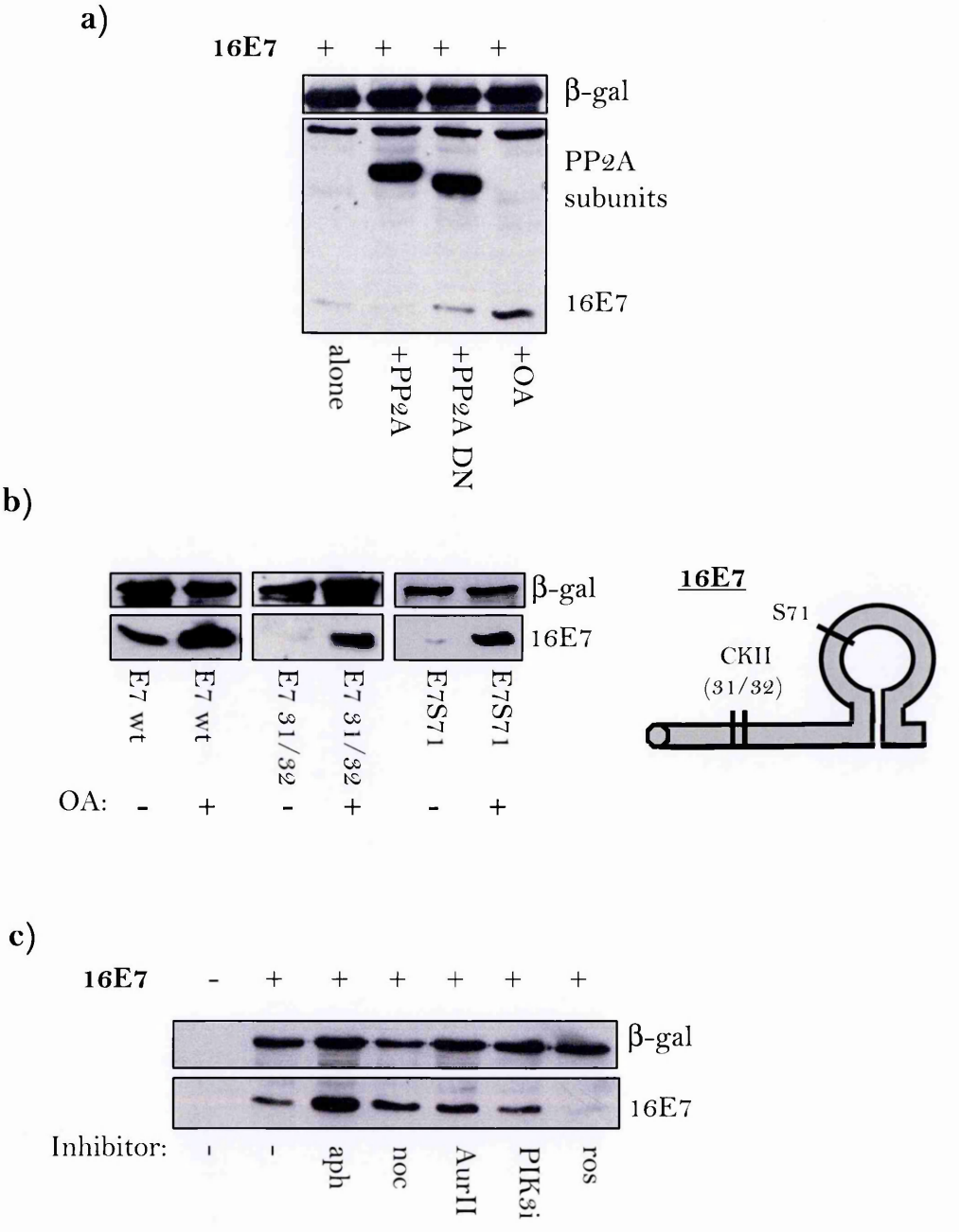


Figure 19. Regulation of E7 stability by phosphorylation.
a) HA-tagged E7 was expressed in U2OS cells along with HA-tagged PP2A or a PP2A dominant negative (DN) mutant. Cells expressing E7 alone were also treated with okadaic acid (OA) for 5hrs prior to harvest. The Western blot analysis shows the levels of HA-tagged proteins using monoclonal anti-HA antibodies and β-gal is shown as a transfection control. b) The levels of expression of the untagged CKII mutant (E7 31/32) and serine 71 mutant (E7 S71) of E7 in the presence of OA compared with untagged wild-type E7 analysed by Western blot using monoclonal antibodies against E7. c) U2OS cells expressing HA-tagged E7 were treated using various kinase and cell cycle inhibitors (see table 3) for 8 hrs. Cells were then harvested and the levels of E7 were analysed by Western blot using anti-HA antibodies.

Table 3: Various Inhibitors used						
Inhibitor name	Aphidicolin	Nocodazole	Roscovitine	Apigenin	LY294002	Aurora kinase II inhibitor
Targeted molecule	DNA polymerase	Microtubule	CDK1/2	CKII	PIK3	Aurora II
Short Name	aph	noc	ros	api	PIK3i	AurII

We extended our analyses to also investigate whether CKII phosphorylation of E7 can modulate its stability. To do this, we included in our assays the specific CKII inhibitor apigenin. Figure 20a shows that inhibiting the activities of both CDK2 and CKII using roscovitine and apigenin, respectively, results in reduced levels of E7. This reduction in the levels of E7 in the presence of CDK2 and CKII inhibitors can be rescued upon the addition of proteasome inhibitors (Figure 20b) indicating that enhanced degradation of E7 is mediated by the proteasome. To confirm the specificity of the kinase inhibitors used, we treated cells expressing the E7 31/32 mutant with apigenin and roscovitine. As can be seen in Figure 20c, the levels of E7 31/32 are unaffected by apigenin treatment, consistent with its not being phosphorylated by CKII, whilst the addition of roscovitine reduces its levels in a manner similar to that seen with the wild-type protein. Overall, these results suggest that phosphorylation of E7 plays an important role in controlling its stability, and that inhibiting the potential phosphorylation of E7 by CDK2 or CKII results in enhanced proteasome-mediated degradation of E7.

E7 is phosphorylated *in vitro* by CDK2

The above studies indicate that E7 may be a potential substrate for phosphorylation by CDK2. To test this, we performed an *in vitro* phosphorylation assay by incubating GST-16E7 with purified CDK2, in the presence of

radiolabelled ATP (^{32}P-ATP), at 30°C for 20 minutes. The amount of ATP incorporation was monitored by auto-radiography and the results are shown in Figure 21a. It can be seen that E7 is significantly phosphorylated by CDK2, mostly on its N-terminal half, compared with the negative control of GST alone.

HPV-16E7 has been shown to form part of CDK2-containing complexes *in vivo* (He et al, 2003) and our results indicate that CDK2 can potentially phosphorylate E7. Since inhibiting CDK2 activity reduces the expression levels of E7 (Figure 19c), ecptopically expressing CDK2 should lead to enhanced levels of E7. To test this, we transiently co-expressed E7 with CDK2 or cyclin E in U2OS cells and analysed E7 protein levels by Western blotting. Figure 21b shows that E7 protein levels are greatly increased in the presence of CDK2 or cyclin E, which confirms that the activity of CDK2, and its potential phosphorylation of E7, plays an important role in regulating the stability of E7. In addition, to further confirm these results, we monitored the levels of E7 during the cell cycle where the activities of different CDK complexes vary. Cells expressing E7 were arrested in G1/S-phase by treatment with aphidicolin for 24 hrs. Following release, the cells were harvested at different points of the cell cycle. As can be seen in Figure 21c, the levels of E7 are significantly higher during the G1/S-phase of the cell cycle (confirmed in the FACS analysis), and this correlates with a higher activity of CDK2/cyclin E complex. This suggests that the activity of endogenous CDK2 can affect the levels of E7 protein along the cell cycle.

To test whether the 5th residue of HPV-16 E7 is phosphorylated by CDK2, we made a point mutation substituting the 5th amino acid of E7 with an alanine (E7 T5A) and made a double mutation of both threonines which flank the 6th proline to alanines (E7 T5/7A). When expressed as a GST-fusion protein, both E7 T5A and

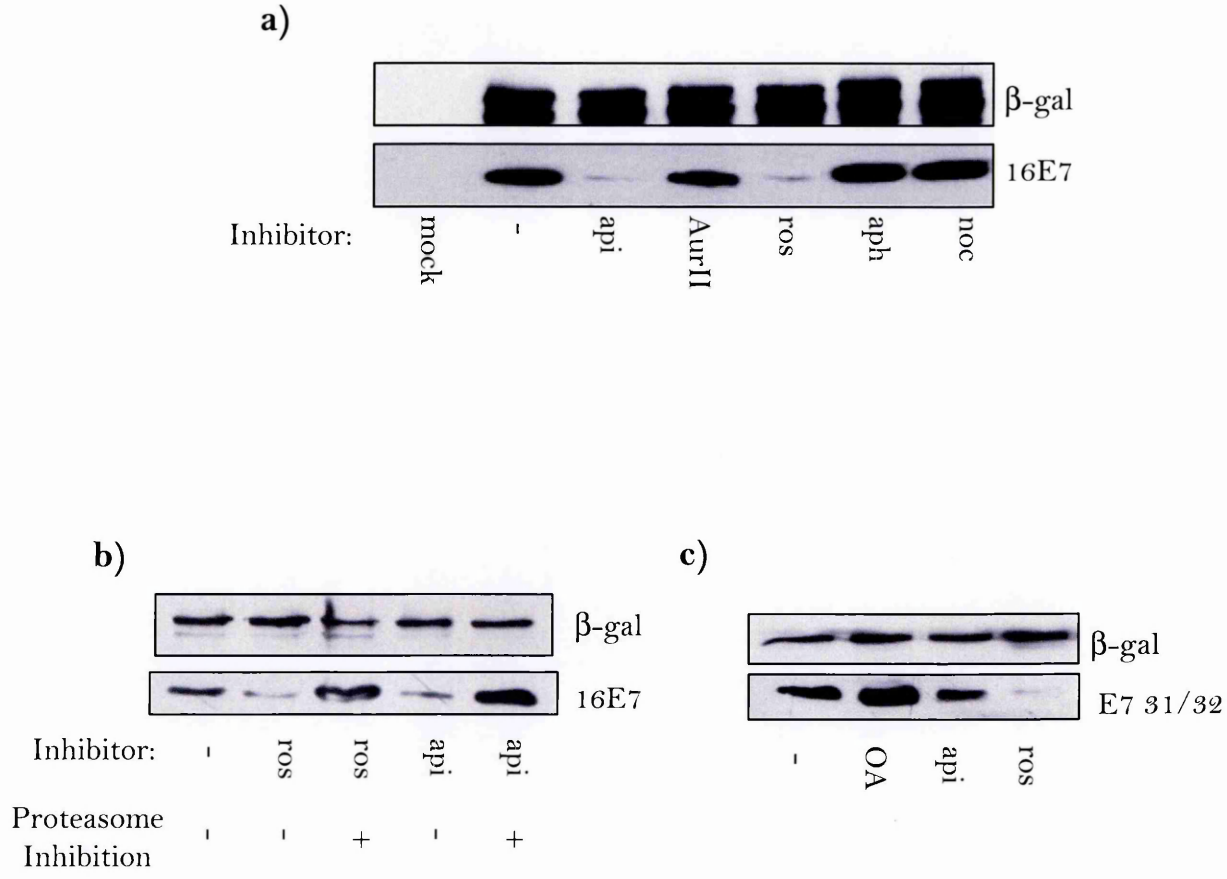


Figure 20. The activities of both CKII and CDK2 are important for regulating proteasome mediated degradation of E7.

a) CKII inhibitors can also affect the stability of E7. U2OS cells expressing pcDNA.16E7 were treated as in 14 (c) along with the inclusion of the CKII inhibitor, apigenin. (api) E7 was detected by Western blot using anti-E7 monoclonal antibodies. b) U2OS cells expressing pcDNA.16E7 were treated for 3 hrs with CDK2 (ros) or CKII (api) inhibitors with or without the addition of the proteasome inhibitor epoxomycin. c) The CKII mutant E7, E7 31/32, is resistant to treatment with apigenin but not to treatment with roscovitine. Cells expressing E7 31/32 were treated for 3 hrs with OA, api or ros, and E7 was detected by Western blot using anti-E7 antibodies.

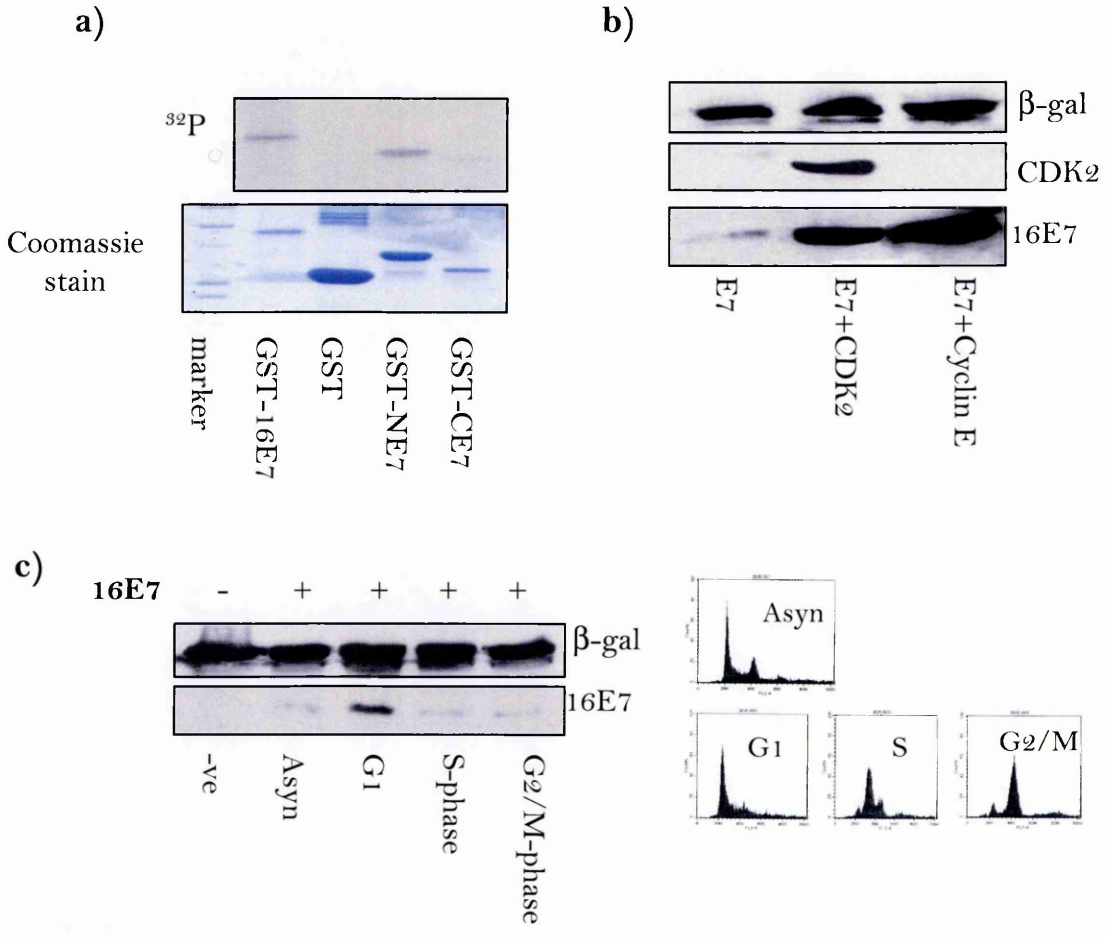


Figure 21. E7 is phosphorylated by CDK2 *in vitro* and the ectopic expression of CDK2 or cyclin E affects its levels *in vivo.*

a) Bacterially purified GST-16E7, as well as the N- and C-terminal halves of E7, were incubated with purified CDK2 along with ATP-^{32}P at 30°C for 20 minutes. After extensive washing with the kinase buffer, the amount of ATP incorporation was analysed using SDS-PAGE and autoradiography. The bottom panel shows the coomassie stain of the input GSTs. b) The over-expression of CDK2 or cyclin E dramatically increases the expression level of E7. U2OS cells were transfected with pcDNA.16E7 expression plasmid, along with HA-tagged CDK2 or a cyclin E expression plasmid. Protein levels were detected by Western blot using anti-E7 and anti-HA antibodies. c) The expression of E7 at different stages of the cell cycle the cell cycle. U2OS cells expressing HA-tagged E7 were treated with aphidicolin for 24 hrs, and then released and harvested at different points in the cell cycle. The levels of E7 expression were analysed by Western blot using anti-HA antibodies. The FACS analyses are also shown to indicate the stage of the cell cycle at which the E7 protein levels were analysed.

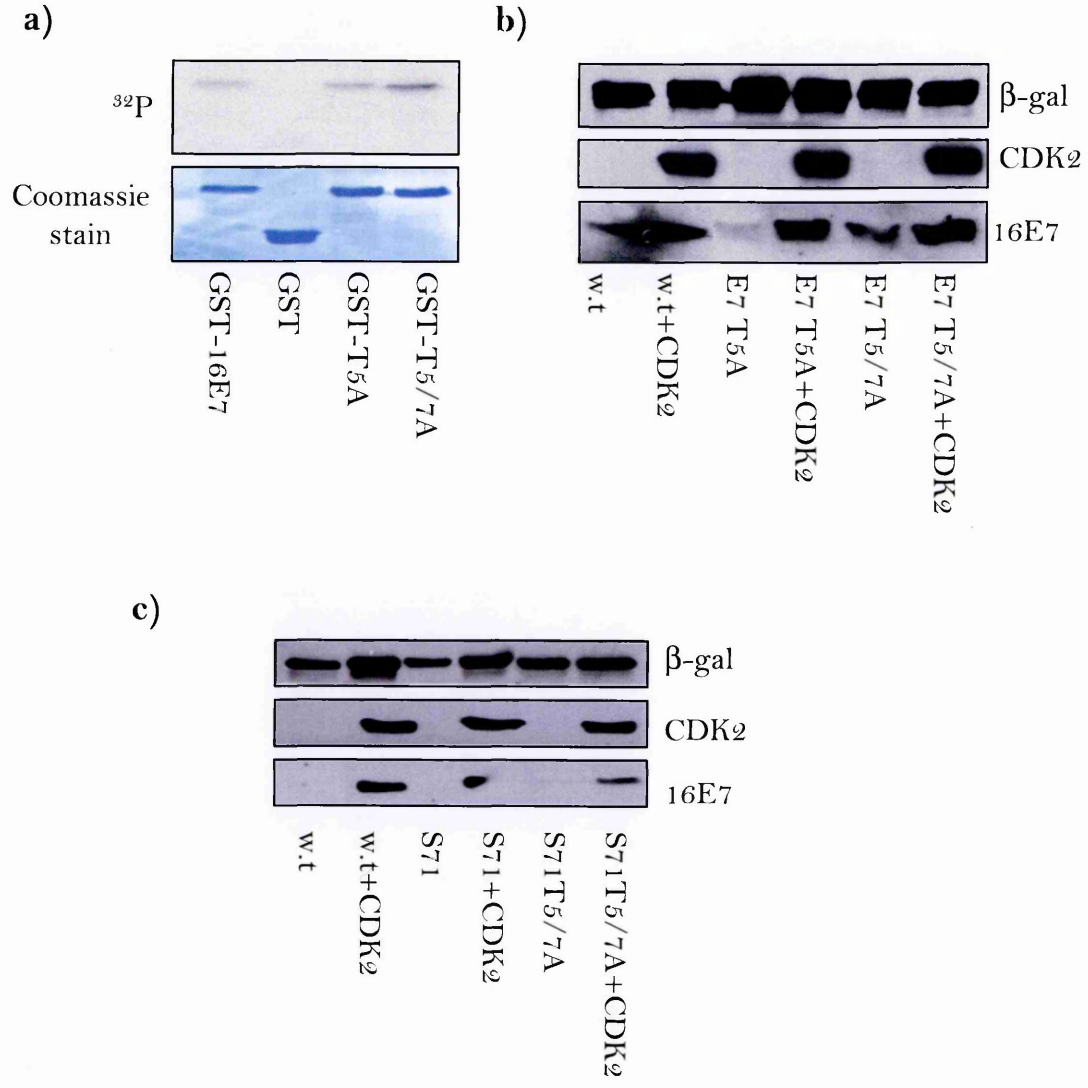


Figure 22. The T5A and the T5/7A mutants of E7 are still phosphorylated by CDK2 *in vitro*.

a) *In vitro* phosphorylation assay using GST-tagged mutants of E7 was conducted as in figure 16a. b) CDK upregulates the expression level of the T5A and T5/7A mutants of E7. Protein levels were detected by Western blot using antibodies against 16E7, HA or β-gal which was used as a transfection control. c) The assay was carried out as in (b) using the E7 S71 or E7 S71T5/7A mutants of E7.

E7 T5/7A were still phosphorylated by CDK2 *in vitro* (Figure 22a). As expected, both mutants of E7 were also stabilised by CDK2 when expressed *in vivo* (Figure 22b), indicating the existence of additional sites for CDK2 phosphorylation that are required for the stabilisation of E7. In addition, we were interested in testing the effects of CDK2 expression on the serine 71 mutation of E7 (E7 S71) which was shown to be phosphorylated by an unknown kinase in a cell cycle dependent manner (Massimi & Banks, 2000). Figure 22c shows that the expression levels of E7 S71, as well as a triple mutant containing substitutions in residues 5,7 and 71 (E7 S71T5/7A), are increased in the presence of CDK2. Overall, these results suggest that CDK2-mediated phosphorylation and stabilisation requires sites additional to threonines 5 and 7 and serine 71 on E7.

A potential role for Mdm2 in regulating HPV protein function

Mdm2 binds to HPV-16 E2 and E7

The proteasome plays an important role in influencing the stability of both E2 and E7 (Bellanger et al, 2001; Reinstein et al, 2000) as well as mediating many of E6 and E7's activities (Boyer et al, 1996; Gardiol et al, 1999; Matlashewski et al, 1987). In addition, E2 was shown to modulate the proteasome-mediated degradation of some of the cellular targets of E6 (Grm et al, 2005) and E7 (this study). We therefore considered to examine whether E2 can interact with the proteasome machinery, and in particular, with Mdm2 which seems a good candidate as it enhances the degradation of both p53 and pRb (Ying & Xiao, 2006), the main cellular targets of E6 and E7, respectively. To address this, we initially sought to

examine whether E2 can interact with Mdm2. Using an *in vitro* binding assay, bacterially expressed GST-fusion proteins were was incubated with *in vitro* translated and radiolabelled Mdm2. Figure 23 shows that Mdm2 can bind to GST-16E2 albeit less strongly than it binds to GST-p53; no binding is seen with GST alone. Unexpectedly, we also detected binding between Mdm2 and GST-16E7; although this was weaker than the E2-Mdm2 interaction, it was, nonetheless, significant. These results indicate a possible interplay between HPV proteins and Mdm2.

Mdm2 enhances proteasome mediated degradation of E7

Since Mdm2 mediates the degradation of a number of its cellular targets, we were first interested in testing the protein levels of E2 and E7 in the presence of Mdm2. To address this, U2OS cells were transfected with E2 or E7 expression plasmids, along with Mdm2 and β-gal as a transfection control. The expression levels of E2 and E7 were then analysed by Western blot. As can be seen in Figures 24a and 27a, increasing the expression of Mdm2 results in a decreased abundance of the E7 protein, while the levels of E2 remain largely unchanged with respect to the β–gal loading control. In the case of E7, its reduced level in the presence of Mdm2 was reversed upon the addition of proteasome inhibitors (Figure 24a), suggesting that Mdm2 targets E7 for proteasome-mediated degradation. These results were further verified using immunofluorescence staining, and it can be seen that the nuclear staining of E7 was abolished in the presence of Mdm2 (Figure 24b, left panel). Interestingly, treating cells with proteasome inhibitors not only resulted in higher levels of E7 in the presence of Mdm2, but also led to the appearance of

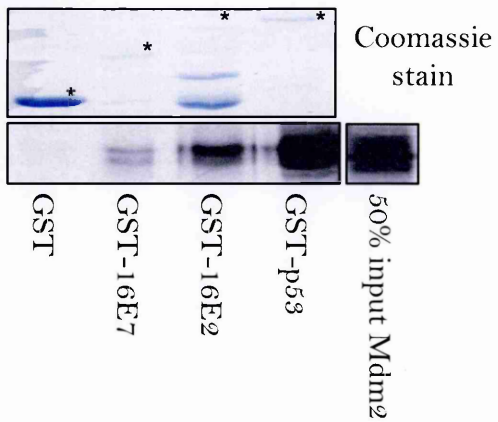


Figure 23. Mdm2 binds to E2 and E7 *in vitro*.
In vitro translated and radiolabelled Mdm2 was incubated with bacterially purified GST-tagged E2 or E7 proteins. GST-alone and GST-p53 were included as negative and positive controls, respectively. The binding reaction was incubated on ice for 1hr, and GST proteins were washed extensively using a detergent containing buffer. Bound proteins were analysed using SDS-PAGE and autoradiography. Coomassie stain of the GST inputs is also included in the upper panel. (*) indicates the full-length GST-fusion proteins.

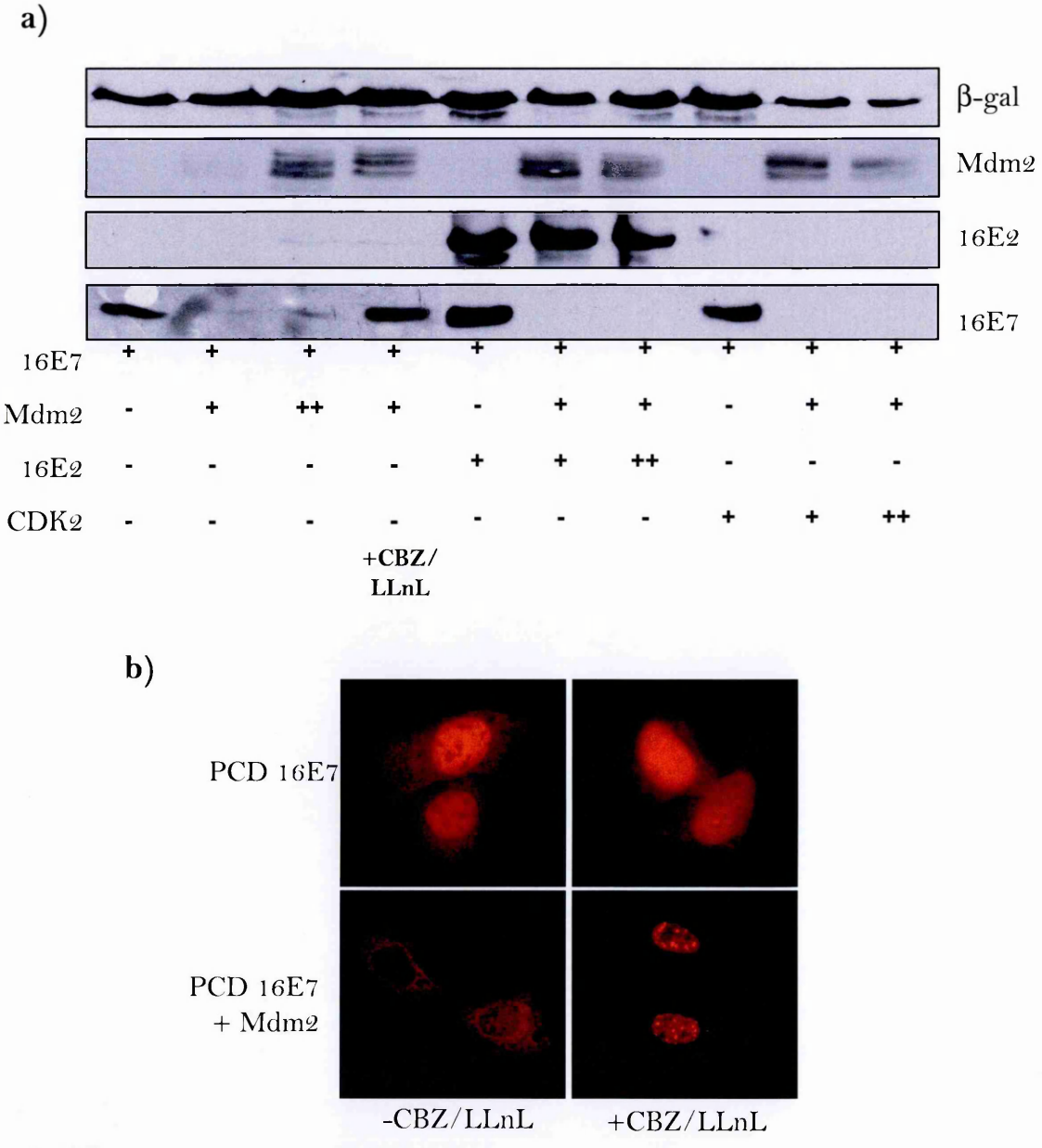


Figure 24. Mdm2 enhances the proteasome mediated degradation of E7.
a) U2OS cells were transfected with a combination of pcDNA.16E7, Mdm2, CDK2 ad 16E2 expression plasmids in the combination indicated. 24hrs after transfection, treatment with proteasome inhibitors CBZ and LLnL (CBZ/LLnL) was conducted for 3hrs, and protein expressions were analysed by Western blotting using antibodies against 16E7, 16E2, Mdm2 and β-gal as a transfection control. b) U2OS cells were transfected with 16E7 alone or with an Mdm2 expression plasmid. In the case of proteasome inhibition, cells were treated with proteasome inhibitors (CBZ/LLnL) for 3hrs prior to fixation. The expression of E7 was analysed by immunofluorescence using antibodies against 16E7.

nuclear domains that are absent in cells that express E7 alone (Figure 24b, right panel). Having shown previously that both E2 and CDK2 can stabilise E7, we further sought to test whether the expression of each protein can compete with Mdm2 in controlling the expression levels of E7. Figure 24a shows that the increasing levels of either E2 or CDK2 did not prevent Mdm2-mediated degradation of E7, suggesting that degradation of E7 by Mdm2 is not affected by its re-localisation with chromatin-bound E2, nor by its increased phosphorylation by CDK2.

To further confirm the Mdm2-mediated degradation of E7, we expressed E7 in U2OS cells with siRNA that specifically targets Mdm2 transcripts (siMdm2). 48 hrs after transfection, the cells were harvested and protein levels were detected by Western blot. To confirm that endogenous Mdm2 protein levels were reduced in the presence of siMdm2, we compared the levels of p53, a well-known target of Mdm2, in cells transfected with siRNA against Luciferase (siLuc) with those transfected with siMdm2. As expected, siMdm2 increased the protein levels of p53 when compared with the control transfection with siLuc (Figure 25a). Interestingly, the level of transfected E7 was also increased upon the knockdown of Mdm2 by siMdm2, suggesting that E7 levels can be controlled by endogenous Mdm2. In addition, we tested the half-life of E7 in mouse embryo fibroblast (MEF) cells that are both Mdm2 and p53 knockout (Mdm2-/- p53-/-) and compared it with the half-life of E7 co-expressed with Mdm2 in the same cells. Figure 25b, left panel, shows that E7 has a prolonged half-life in Mdm2-/- p53-/- cells of up to 2 hrs, which far exceeds the already established half-life of E7 in wild-type MEF cells of about 30 minutes (Oh et al, 2004a). Interestingly, the half-life of E7 is markedly reduced to about 15 minutes in the presence of Mdm2 (Figure 25b, right panel),

resembling that of E7 expressed in U2OS (Figure 10e). Overall, these results provide evidence that Mdm2 can radically decrease the stability of E7.

E7 is targeted for degradation by Mdm2 at the sites of nuclear domains

We were further interested in characterising the nuclear domains that appear as a result of co-expressing Mdm2 and E7 in the presence of proteasome inhibitors. Using immunofluorescence staining we can confirm that, in the presence of proteasome inhibitors, Mdm2-mediated degradation of E7 is inhibited, and that both proteins co-localise in nuclear domains (Figure 26a). Since it has been shown previously that PML bodies can act as sites of nuclear protein sequestration, we used polyclonal antibodies against endogenous PML proteins to show that these nuclear domains are also sites of PML protein localisation (Figure 26b). In addition, by staining cells with FK2 antibodies, which specifically recognise protein-bound but not free ubiquitin, these nuclear domains were found to be highly enriched in ubiquitinated proteins (Figure 26c). This demonstrates that inhibiting Mdm2-mediated degradation of E7 results in formation of nuclear domains that are rich in ubiquitinated proteins and where Mdm2, E7 and PML bodies co-localise, suggesting that Mdm2 mediates the degradation of E7 at specific sites in the nucleus.

The interaction between Mdm2 and E2 results in the increased transcriptional activity of E2

Since we observed no change in E2 levels in the presence of Mdm2 (Figure 27a), we sought to examine whether there are any potential effects upon each other's

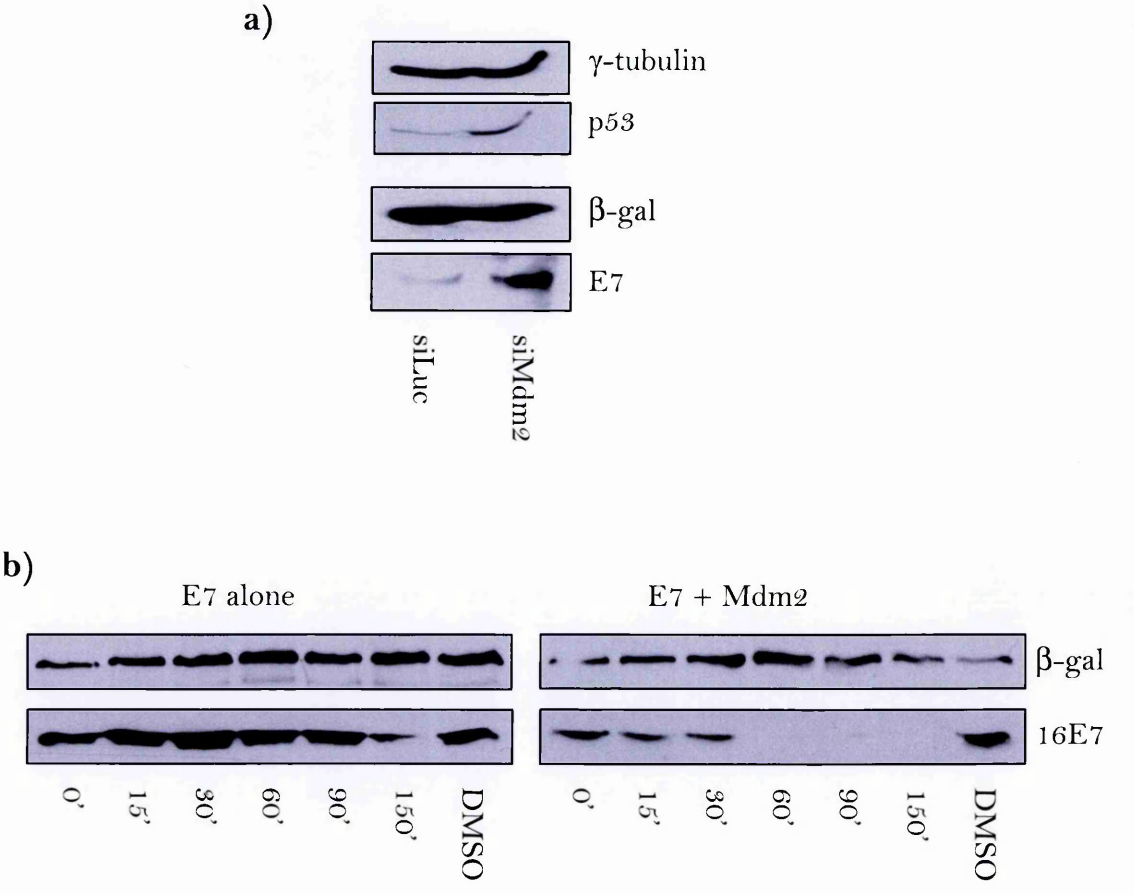


Figure 25. The knockdown of Mdm2 expression increases the stability of E7. a) siRNA knockdown of endogenous Mdm2 expression results in increased levels of E7. U2OS cells were transfected using Lipofectamine2000 with pcDNA.16E7 and β-gal along with control siRNA or siRNA against Mdm2 (siMdm2). 48 hrs after transfection, protein levels were detected by Western blot using an antibody against E7 or β-gal as a transfection control. b) The half life of E7 is prolonged in Mdm2-/- p53-/- MEF cell line. Cells were grown in 6-well plates, and transfected using Lipofectamine2000 with pcDNA.16E7 alone or with Mdm2 expression plasmid. 24 hrs after transfection, cells were treated for different times (0, 15, 30, 60, 90 and 150 minutes) with cycloheximide in DMSO or with DMSO alone for 150 minutes. Cells were then harvested and the protein levels of E7 were analysed by Western blot using monoclonal antibodies against 16E7 or β-gal as a marker for transfection efficiency.

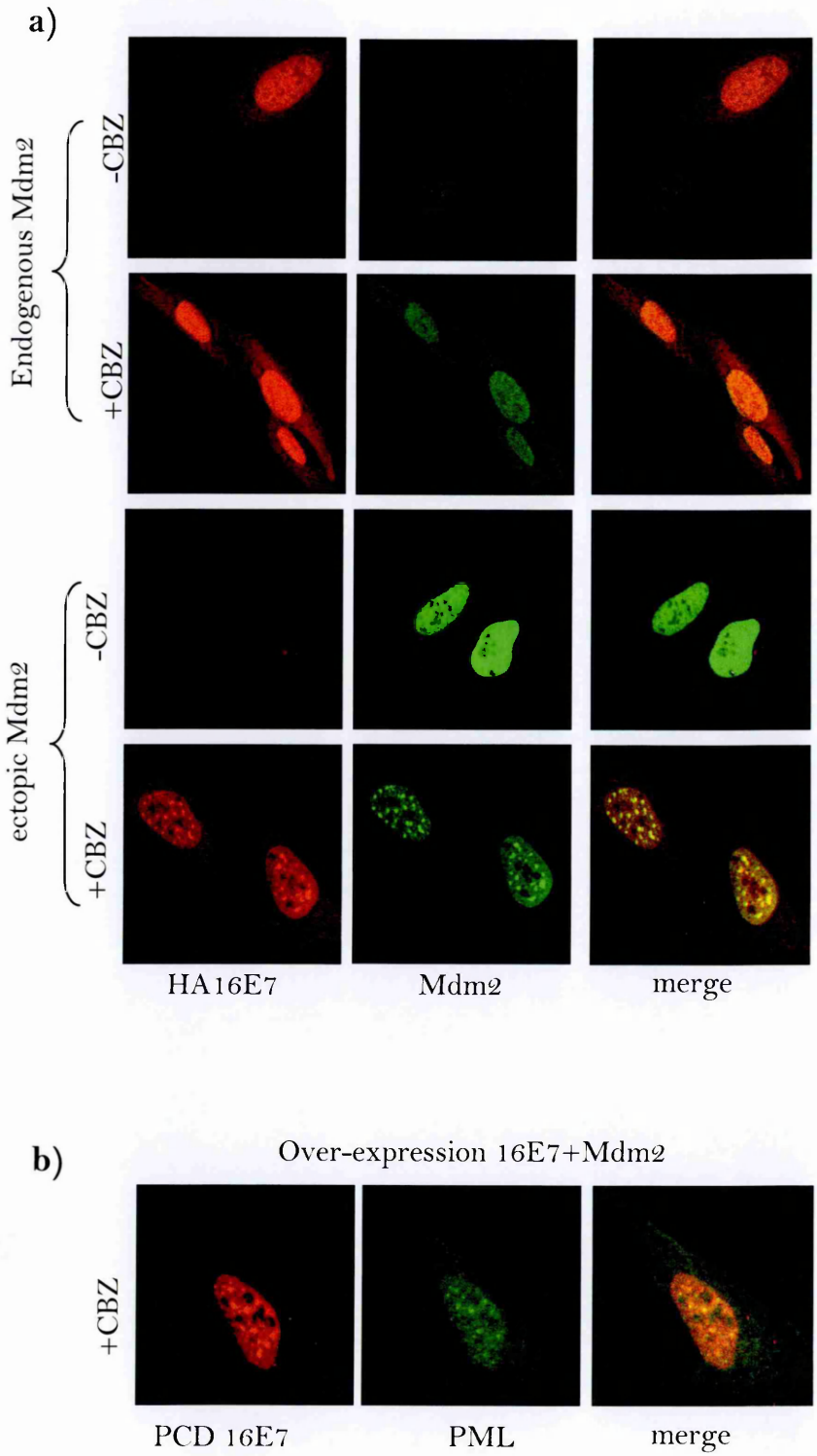
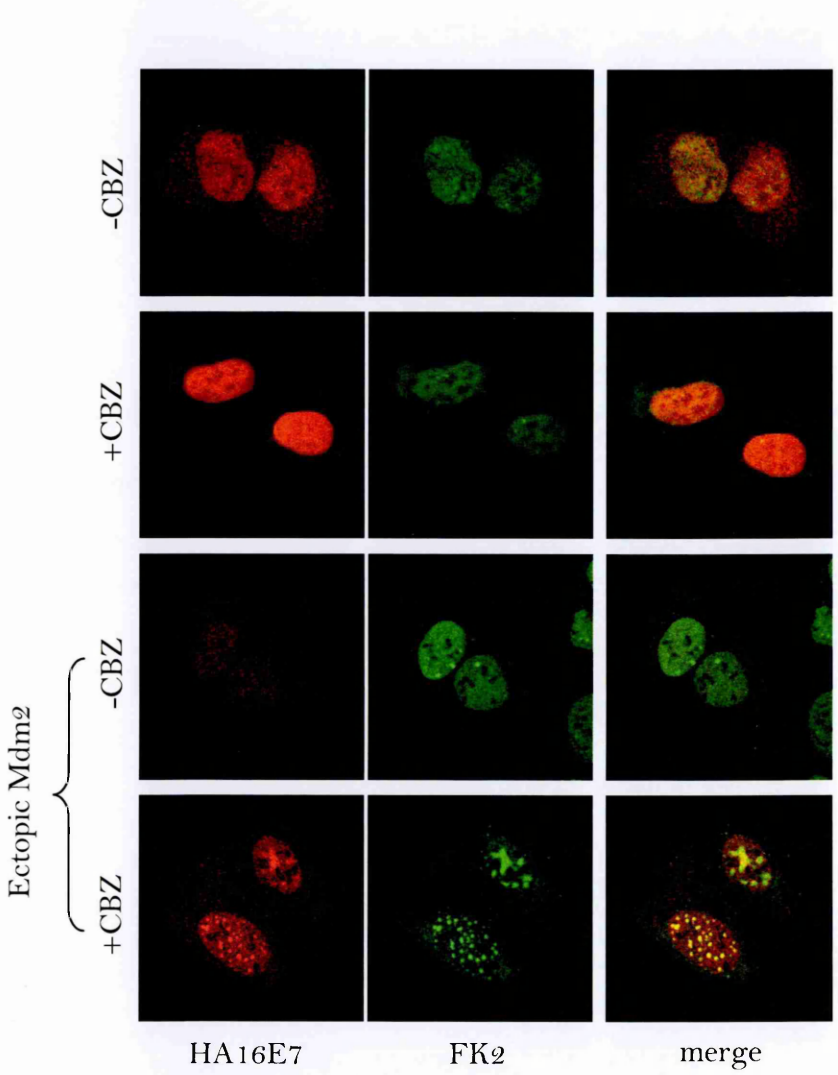


Figure 26. Mdm2 mediates the relocalisation of E7 into ubiquitin rich nuclear domains.

U2OS cells expressing HA-E7 alone, or HA-E7 with Mdm2 were left untreated or treated with proteasome inhibitors (CBZ) for 3hrs prior to fixation. Cells were then stained using the following combinations of antibodies; rabbit anti-HA and mouse anti-Mdm2 (a); mouse anti-HA and rabbit anti-PML (b); rabbit anti-HA and mouse anti-FK2 (c). The results obtained are representative of at least 3 independent experiments.

Figure 26 (*cont.*)



activities. In order to verify that E2 and Mdm2 can form a complex *in vivo*, we performed a co-immunoprecipitation assay from 293 cells that are transfected with either Mdm2 alone or Mdm2 with GFP-tagged E2. Immunoprecipitation was conducted using antibodies against GFP to pull down E2 followed by Western blot analysis using an anti-Mdm2 monoclonal antibody to detect immunoprecipitated proteins. Figure 27b shows that a complex between Mdm2 and E2 can be detected in cells expressing Mdm2 and E2, but not in cells expressing Mdm2 alone. Furthermore, to identify the region of E2 that mediates its interaction with Mdm2, we used a number of GST-tagged E2 fragments, including the N- and C-terminal domains and truncation fragments of the C-terminal domain (see Figure 7a). Figure 27c shows the results of an *in vitro* binding assay between the GST-tagged E2 fragments and *in vitro* translated Mdm2. It can be seen that E2 binds to Mdm2 mainly through its C-terminal half and, more specifically, through a region spanning residues numbers 322-335. Since the C-terminal domain of E2 mediates its DNA binding, we sought to test whether Mdm2 can affect the transcriptional activity of E2. To do this, we performed a luciferase reporter assay using a construct that contains 6 synthetic tandem repeats of the E2 DNA-binding site upstream of the luciferase gene (see Figure 15a). This assay was performed in both U2OS and SAOS-2 cells to rule out the effects of p53 and pRB inactivation induced by Mdm2. The cells were transfected with the reporter plasmid together with GFP-tagged E2 and Mdm2 expression plasmids and luciferase activity was assayed 24 hrs after transfection. As can be seen in Figure 27d, the expression of Mdm2 strongly enhances the transcriptional activity of E2 in both cell lines, showing that Mdm2 enhances E2's transcriptional activity in a manner independent of p53 and pRB inactivation by Mdm2.

The expression of E2 inhibits Mdm2-mediated degradation of p53 and pRB

To test the effects of E2 on the activities of Mdm2, we then examined the ability of Mdm2 to degrade p53 and pRB in the presence of E2. This was done by transiently expressing FLAG-tagged p53 in U2OS cells and pRB in SAOS-2 cells, together with Mdm2 and E2 as indicated. Figures 28a&b show that E2 can strongly inhibit Mdm2-mediated degradation of both pRB and p53. In the absence of Mdm2, E2 expression has no effect on pRB levels, but results in an increase of p53 levels in both soluble and insoluble fractions of the cell extract (Figure 28b). This could be the result of the previously described interaction between p53 and E2 (Massimi et al, 1999). These results suggest that E2 can inhibit Mdm2 mediated degradation of both pRB and p53.

The activity of E2 in opposing Mdm2-mediated degradation of its substrates was further confirmed using immunofluorescence. Both p53 and E2 are diffused in the nucleus when co-expressed in untreated U2OS cells or in cells treated with proteasome inhibitors (Figure 29a). Upon the addition of Mdm2 expression plasmid, the expression of p53 is still detected, thereby confirming the Western blot analysis in Figure 28b. In these cells however, both p53 and E2 become less diffused in the nucleus even in the absence of proteasome inhibitors (Figure 29b, upper panel). Treating these cells with proteasome inhibitors, E2 and Mdm2 form more discrete nuclear domains (Figure 29b, lower panel).

We were then interested in characterising the nuclear structures that form in cells treated with proteasome inhibitors and express Mdm2. First, we show that in the absence of Mdm2, E2 has a diffused nuclear staining that remains unchanged in the presence of proteasome inhibitors (Figure 30a). The additional expression of Mdm2 does not greatly affect the localisation of E2 (Figure 30b, upper panel),

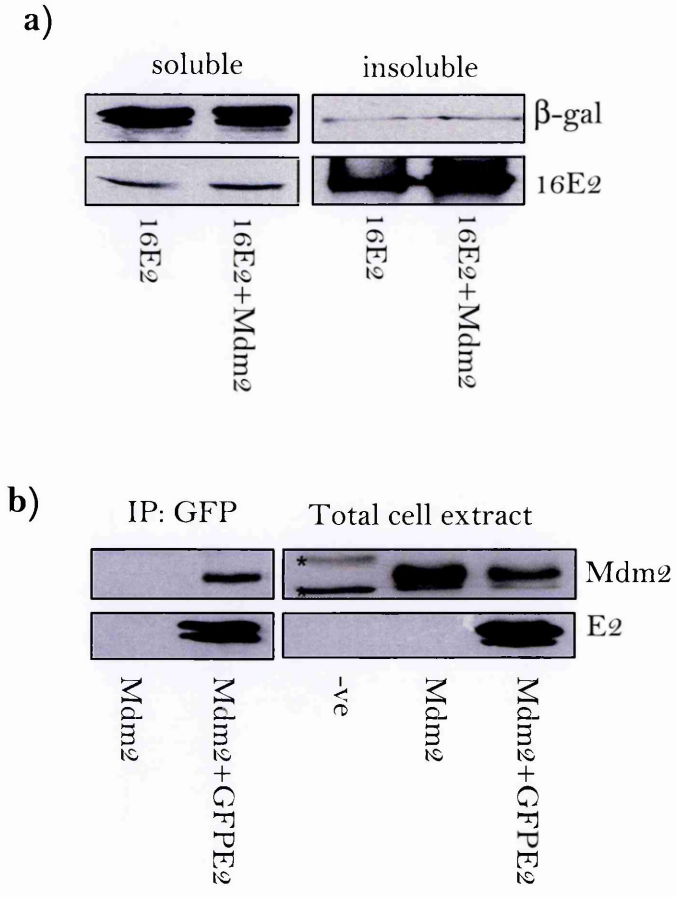
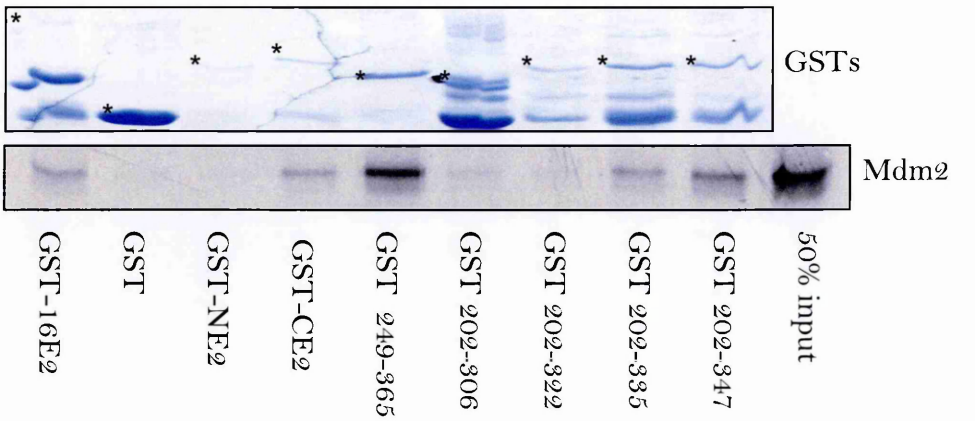


Figure 27. Mdm2 interacts with 16E2 both *in vitro* and *in vivo*.
a) The effect of Mdm2 expression on E2 levels was tested by expressing both proteins in U2OS cells. 24hrs after transfection, protein levels were analysed, in both the soluble and insoluble fraction of the cell, by Western blot using antibodies against 16E2 or β-gal as a transfection control. b) E2 and Mdm2 bind *in vivo*. 293 cells were transfected using either Mdm2 alone or Mdm2 with GFP-tagged E2. Cell extracts were immunoprecipitated using anti-GFP antibodies followed by Western blot analysis using antibodies against Mdm2 or GFP. (*) indicate non-specific bands.

c)



d)

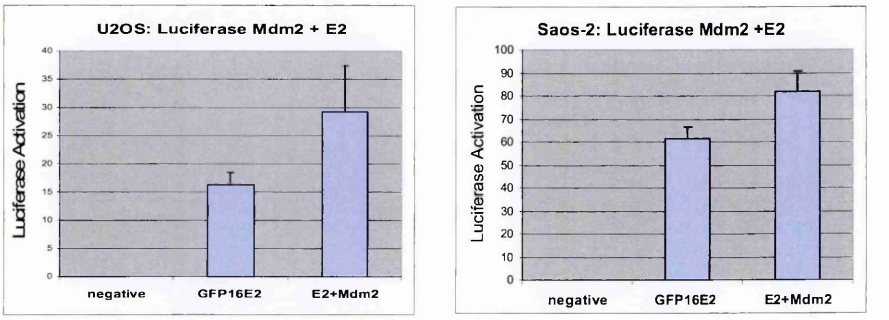


Figure 27. Mdm2 interacts with 16E2 both *in vitro* and *in vivo.* (*cont.*)
c) Mdm2 binds to the C-terminal of E2. GST-16E2 and a number of GST-tagged fragments of E2 (figure 7a) were incubated with *in vitro* translated and radiolabelled Mdm2. Binding reactions were incubated for 1 hr on ice, and bound proteins were analysed using SDS PAGE and autoradiography. 50% of the input is included and the GST inputs are shown below stained with coomassie where (*) indicate the full-length GSTs. d) Mdm2 enhances E2-mediated transcriptional activity. U2OS and SAOS-2 cells were transfected with reporter construct containing 6X E2 binding sites upstream the luciferase gene, plus the *Renilla* luciferase gene as a transfection control and a GFP-E2 expression vector with or without the expression of Mdm2. Representative results of three experiments are shown together with standard deviation.

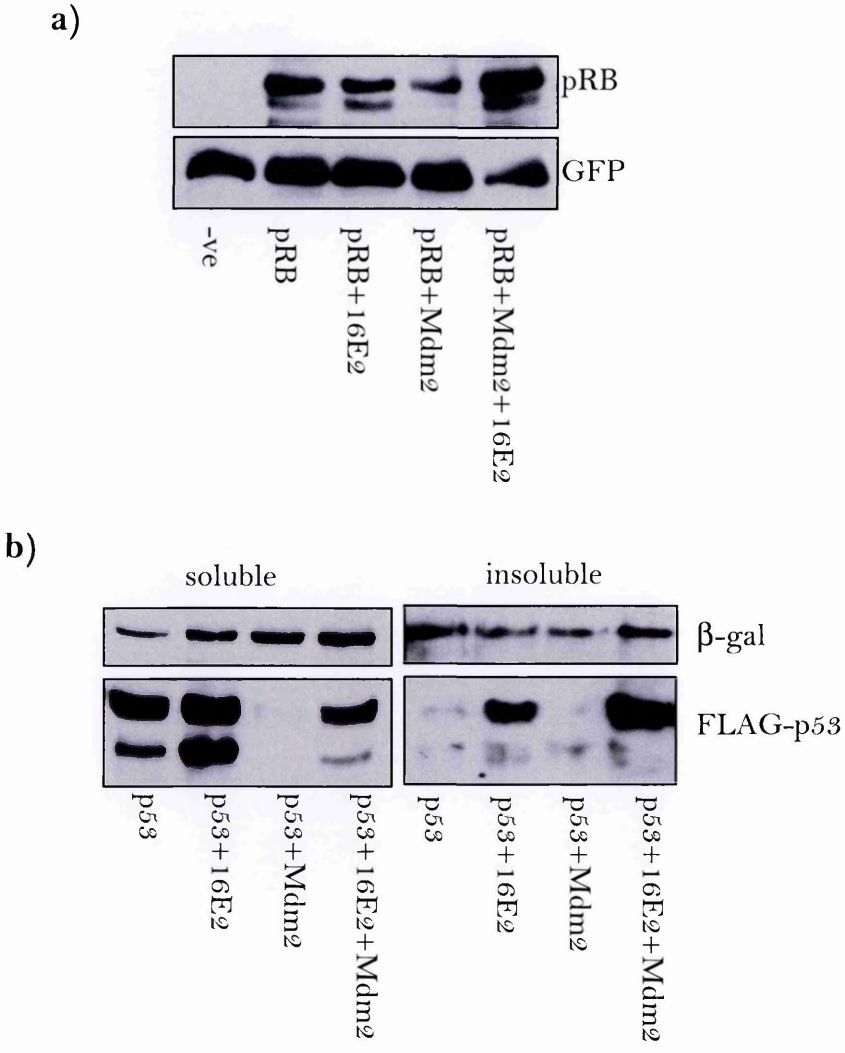


Figure 28. E2 inhibits Mdm2 mediated degradation of pRB and p53.
a) SAOS-2 cells were transfected with a pRB expression plasmid, along with a combination of 16E2 and Mdm2 expression plasmids as indicated. Proteins levels were assessed by Western blot 24hrs post transfection using antibodies against pRB or GFP as a transfection control. b) p53 degradation by Mdm2 was assessed in U2OS cells by the expression of FLAG-tagged p53 construct along with a combination of E2 and Mdm2 expression plasmids as indicated. The levels of p53 were assessed by Western blot using anti-FLAG antibodies.

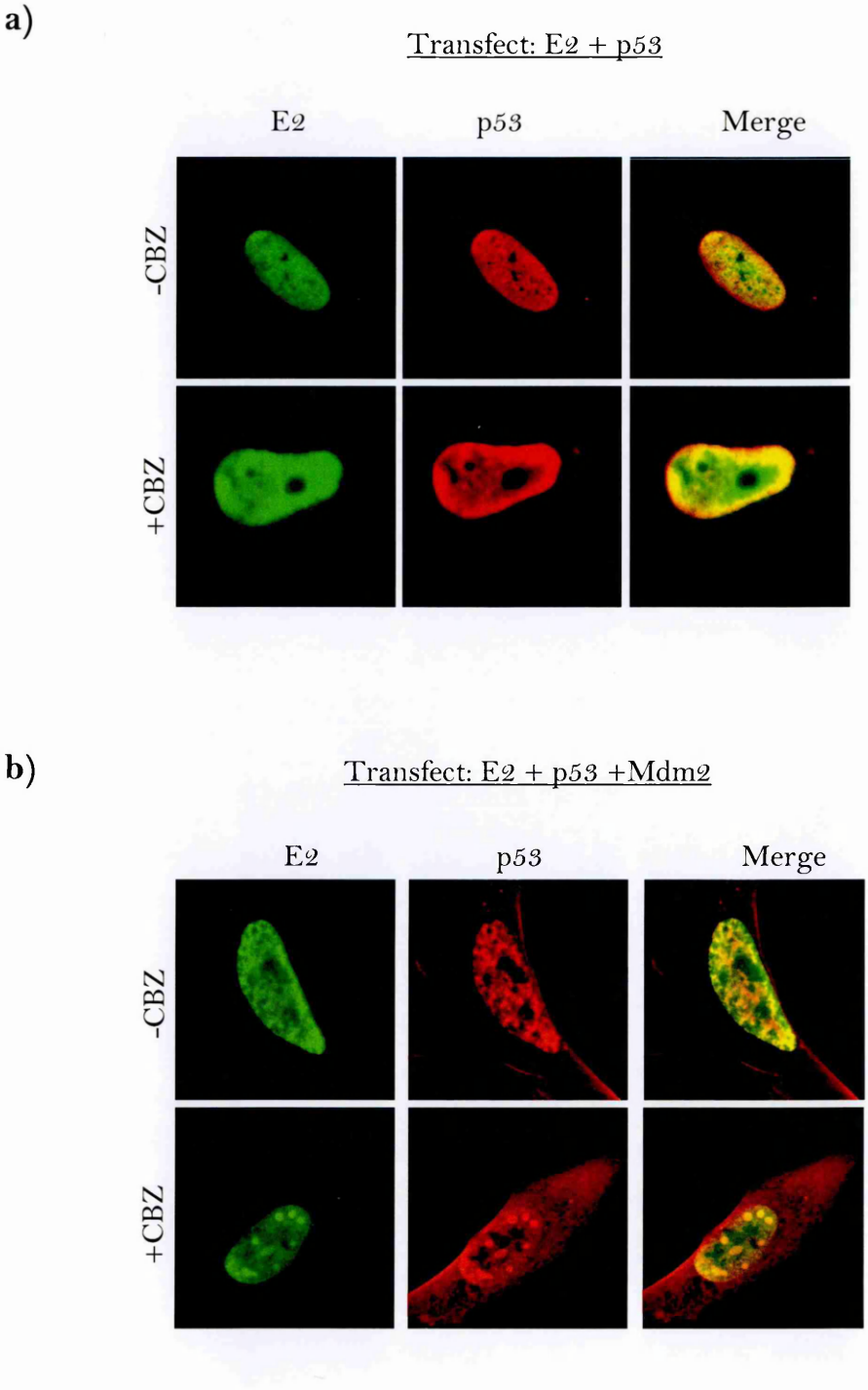


Figure 29. E2 is localised with p53 in specific nuclear structures in the presence of Mdm2.
U2OS cells were transfected with FLAG-tagged p53 and E2 expression plasmids in the absence (a) or the presence (b) of an Mdm2 expression plasmid. Cells were transfected in duplicates and one set was treated with proteasome inhibitors (a&b lower panels). The expression of p53 and E2 was visualised using monoclonal anti-FLAG and polyclonal anti-E2 antibodies. Images were scanned using confocal microscopy. The results obtained are representative of at least 3 independent experiments.

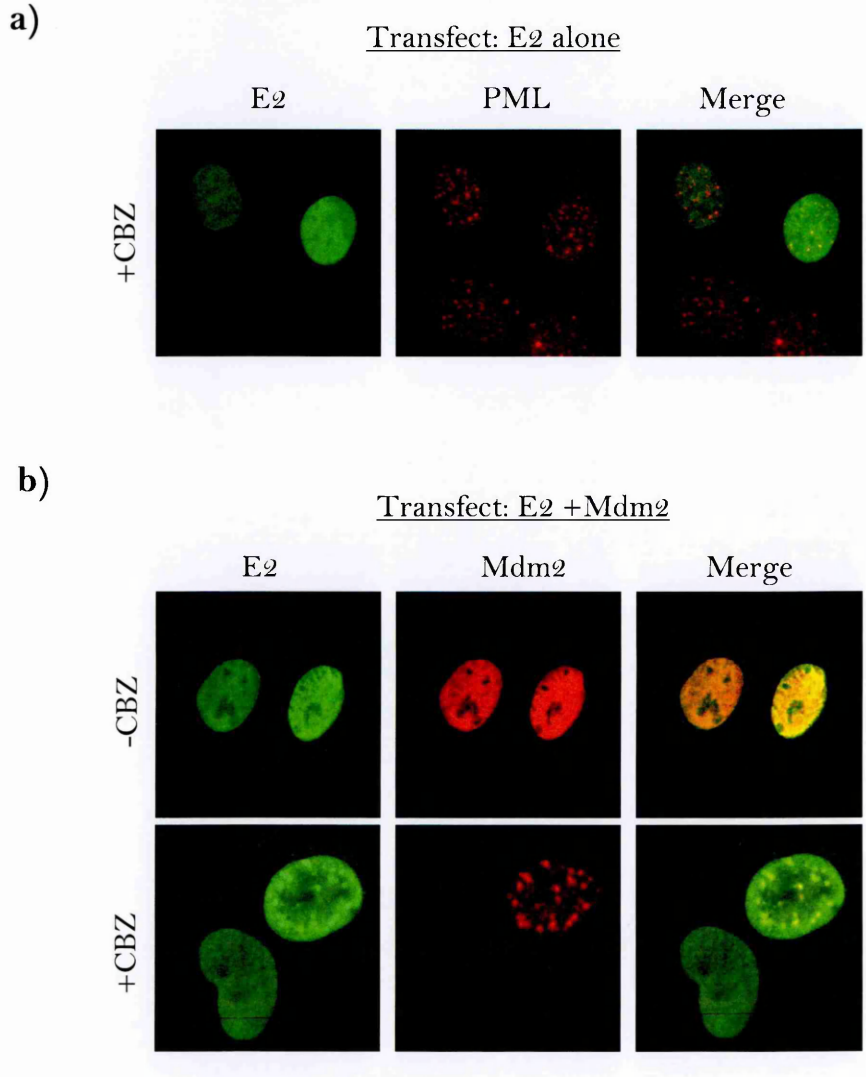
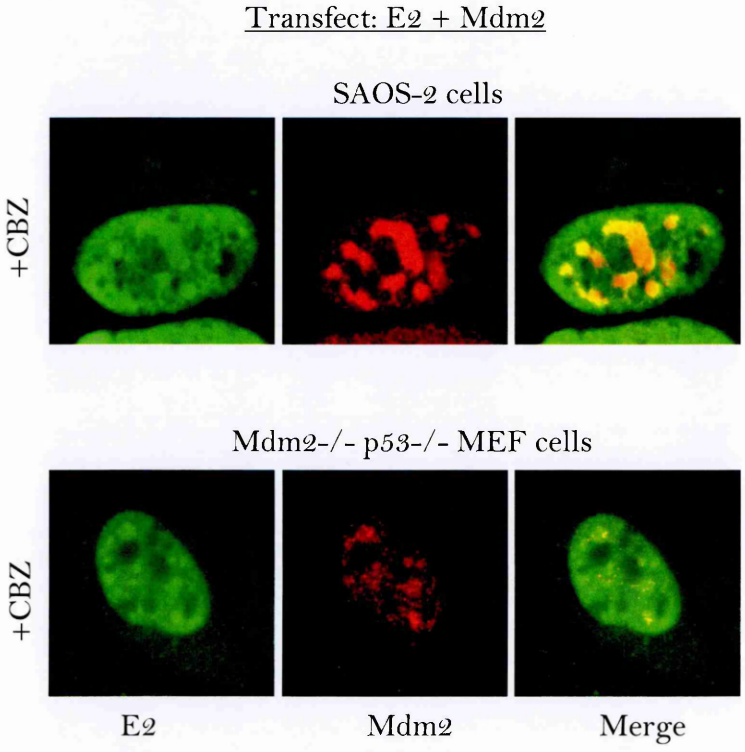


Figure 30. E2 is localised to nuclear structures in the presence of Mdm2
a) The expression of E2 is unchanged in the absence of Mdm2 expression in cells that are treated with proteasome inhibitors. U2OS expressing E2 were stained using monoclonal anti-E2 and polyclonal anti-PML antibodies. b) E2 and Mdm2 co-localise. U2OS cells were transfected with E2 and Mdm2 expression plasmids in duplicates, and one set of transfections was treated with proteasome inhibitors for 3hrs prior to fixation (lower panel). Cells were stained with polyclonal anti-E2 and monoclonal anti-Mdm2 antibodies. c) The relocalisation of E2 with Mdm2 is independent on its interaction with p53. SAOS-2 and Mdm2-/- p53-/- MEF cells were transfected with E2 and Mdm2 expression plasmids. 24 hrs after transfection cells were treated with proteasome inhibitors and stained as in (b). The results obtained are representative of at least 3 independent experiments.

Figure 30 (*cont.*)

c)



however, upon treatment with proteasome inhibitors, cells that express both Mdm2 and E2 form nuclear domains in which both proteins co-localise (Figure 30b, lower panel). Furthermore, to show that the re-localisation of E2 by Mdm2 to these nuclear domains is independent on its binding to p53, we repeated the same experiment and expressed E2 with Mdm2 in p53 negative cells including SAOS-2 and Mdm2-/- p53-/- MEF cells. Figure 30c shows that the relocalisation of E2 by Mdm2 in the presence of proteasome inhibitors is independent on the binding of E2 to p53. Overall, these results show that the expression of Mdm2 is essential for the relocalisation of E2 to specific structures within the nucleus.

The nuclear domains we observe resemble nuclear bodies that assemble as a result of cellular stress induced by the inhibition of the proteasome, and have been previously shown to be sites where PML domains, Mdm2 and nucleoli co-localise (Bernardi et al, 2004; Kurki et al, 2003). They are also sites where the activities of pro-apoptotic proteins such as p53 and Daxx are controlled. To characterise whether the nuclear domains observed in our assays correspond to nuclear stress bodies, cells expressing Mdm2 and E2 were treated with proteasome inhibitors and stained using antibodies against endogenous p53, Daxx, PML, nucleoli (C23 antibody) and ubiquitinated proteins (FK2 antibody). Indeed, Figure 31 shows that upon the expression of Mdm2, E2 co-localises with components of nuclear stress bodies indicating that E2 can regulate Mdm2-mediated degradation of p53 and is localised by Mdm2 to nuclear stress bodies, which contain components of PML bodies (in the case of p53, PML and Daxx) as well as nucleolar structures and are enriched in polyubiquitinated proteins. This confirms that these structures are stress-induced nuclear bodies (Bernardi et al, 2004; Kurki et al, 2003).

Finally, having shown that E2 inhibits Mdm2-mediated degradation of p53 and can co-localise with p53 at ubiquitin-rich nuclear domains, we were interested to investigate whether pRB, is also re-localised to these nuclear domains. In order to investigate this, U2OS cells were transfected with E2, Mdm2 and pRB expression plasmids. The results in Figure 32a show that both pRB and E2 are diffused in the nucleus in untreated cells (upper panel), however in cells that are treated with proteasome inhibitors pRB does not localise with E2 (lower panel) or with Mdm2 (Figure 32b) at nuclear domains. These results indicate that E2 rescues Mdm2-mediated degradation of pRB but does not co-localise with pRB at nuclear domains.

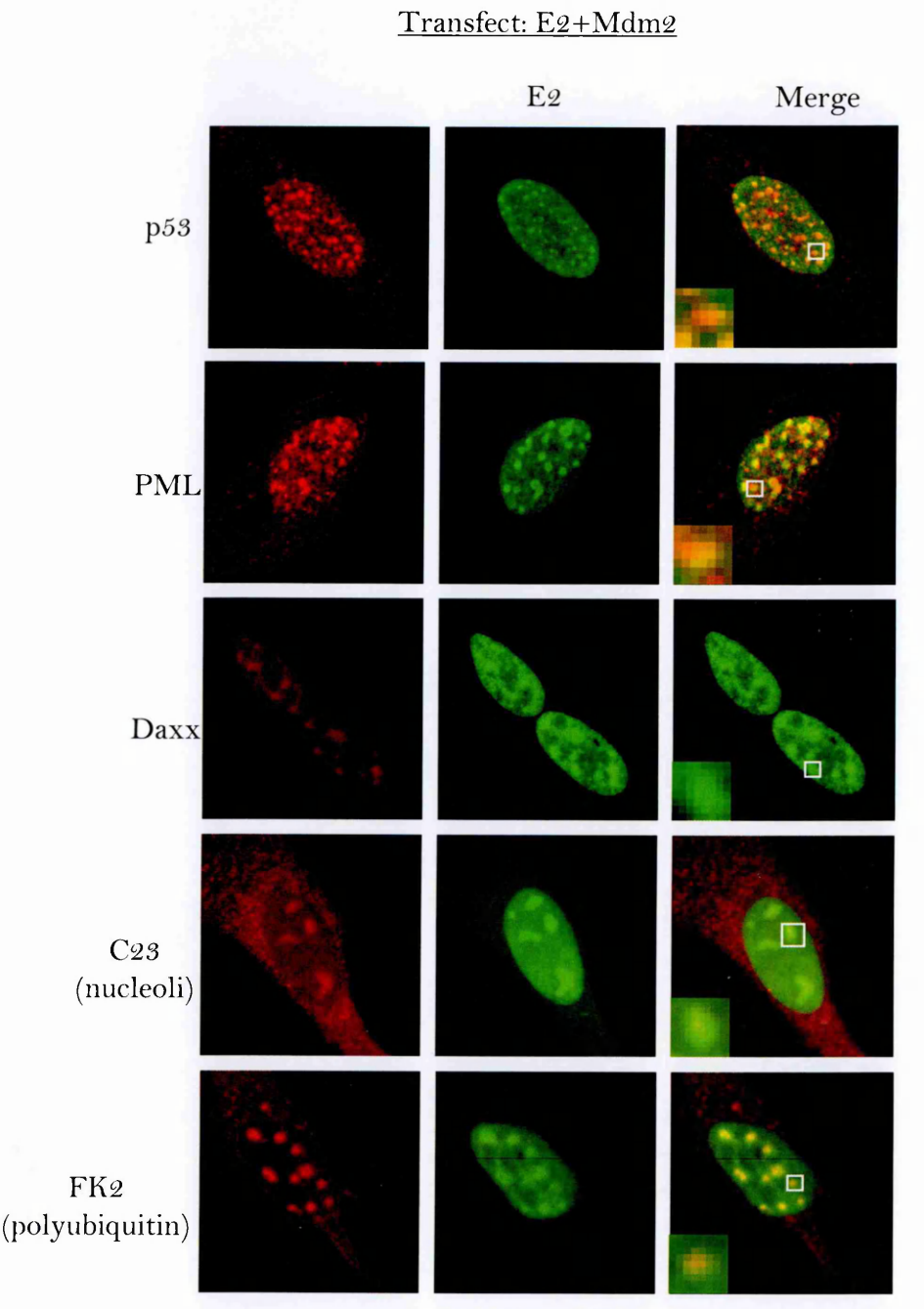
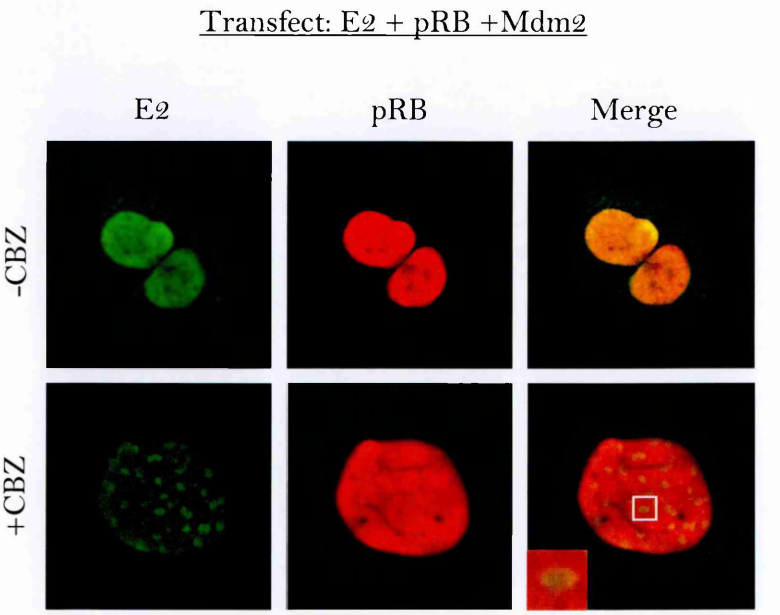


Figure 31. E2 and Mdm2 co-localise in nuclear stress bodies in cells treated with proteasome inhibitors.

U2OS cells were transfected with E2 and Mdm2. 24hrs after transfection, cells were treated with proteasome inhibitors for 3hrs prior to fixation. Anti-E2 antibodies were used to stain cells along with antibodies against endogenous proteins including, Daxx, p53, FK2, C23 and p53. The cross-talk between the green and red channels was avoided by the use of two separate excitation wavelengths as indicated in Materials and Methods. Relevant subcellular structures are labelled with white boxes and enlarged on the lower left side of the merged image. The results obtained are representative of at least 3 independent experiments.

a)



b)

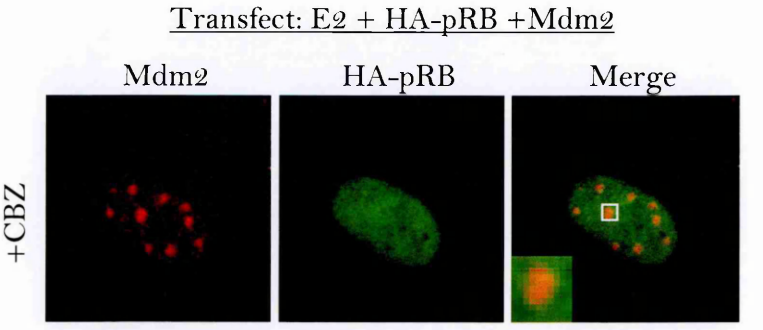


Figure 32. pRB does not localise with E2 and Mdm2 in U2OS cells.
U2OS cells were transfected with E2, Mdm2 and either pRb or HA-tagged pRB expression plasmids. 24hrs, cells were left untreated or treated with proteasome inhibitors as indicated. Cells were then fixed and stained with polyclonal anti-E2 and monoclonal anti-pRB (a) or monoclonal anti-Mdm2 and polyclonal anti-HA (b). Relevant subcellular structures are labelled with white boxes and enlarged on the lower left side of the merged image. The results obtained are representative of at least 3 independent experiments.

Discussion

Unlike lytic viruses, HPVs can replicate their DNA and release infectious virions without causing the death or transformation of their host cells. The virus initially infects the basal layer of the epithelium and the production of mature virions is only observed in the upper granular layer (Howley & Lowy, 2001). With respect to the papillomavirus life cycle, viral genome integration and cellular immortalisation are both disadvantageous for the virus; the replicative capacity of the virus is lost with the loss of the E2 protein and immortalised cells are unable to differentiate into the stratum corneum where mature virions are formed and shed (Figure 3). Therefore, to ensure an efficient and productive life cycle, HPVs finely modulate the activities of cellular proteins and their own viral gene products by means of various interactions. In this study, we have identified several mechanisms involving both viral and cellular gene products that modulate the activities of HPV-16 E7, whose expression is upregulated in cervical cancer (figure 33a). These include direct binding between E7 and the viral transcriptional activator E2, phosphorylation of E7 by CDK2 and CKII, plus interaction of E7 with Mdm2.

Interplay between HPV-16 E2 and E7

Several studies have shown transcriptional regulation of the viral oncoproteins, E6 and E7, by E2 (Cripe et al, 1987; Dowhanick et al, 1995; Goodwin & DiMaio, 2000). Other studies show that E2 can, additionally, control the oncogenicity of E6 and E7 in a transcriptionally-independent manner. Both HPV-16 and -18 E2 proteins can induce apoptosis in HPV-positive cell lines in the absence of their

DNA-binding domains (Blachon & Demeret, 2003; Demeret et al, 2003; Webster et al, 2000). In addition, it has been previously shown that mutations in the E2 binding sites proximal to the p97 promoter do not fully alleviate E2-mediated repression of HPV-16-induced immortalisation of primary human keratinocytes (Romanczuk & Howley, 1992). Overall, these studies provide evidence of a transcriptionally-independent capability of E2 to counteract the activity of the viral oncoproteins. In this study we propose a novel post-transcriptional mechanism by which E2 interferes with the oncogenicity of E7 through direct interaction between the two proteins. This interaction seems to be specific to high-risk and not low-risk HPV types (Figure 6d), thus suggesting its relevance to the life cycle of high-risk HPV types, although we cannot exclude that the interaction between low-risk E2 and E7 may occur at a lower affinity or under certain conditions. The interaction between HPV-16 E2 and E7 is demonstrated using both *in vitro* and *in vivo* binding assays and is supported by a series of functional assays.

Mapping site of interaction between E2 and E7

The site of interaction between the E2 and E7 maps to the middle hinge region of E2 which is highly unconserved between E2 proteins from different HPV types. Only a small number of proteins was shown to bind to this region of E2 from various papillomavirus types, including the binding of HPV-8 E2 to sp1 (Steger et al, 2002) and HPV-5 E2 to SR proteins (Lai et al, 1999). In addition, NLS and CKII phosphorylation sites that affect the localisation of HPV-11 E2 and the stability of BPV-1 E2, respectively, are both located at the hinge region (Penrose et al, 2004; Zou et al, 2000). Interestingly, unlike the DNA binding and dimerisation domains of E2 that are conserved in E2 proteins from various HPV types, protein

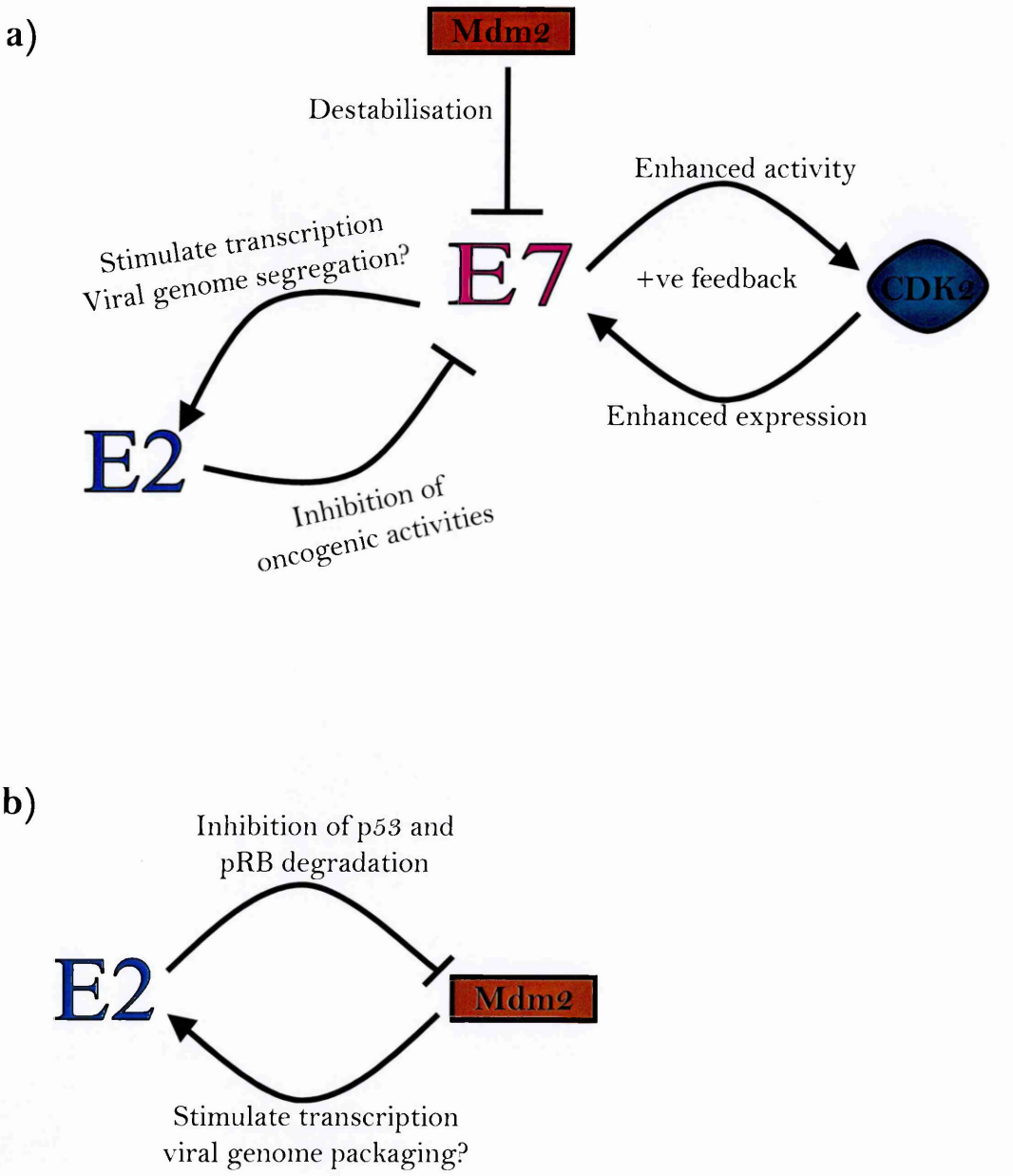


Figure 33. a) Summary of mechanisms regulating HPV16 E7 activities analysed in this study. b) Interplay between HPV-16 E2 and Mdm2. See text for more details.

interactions and modifications of the hinge region are poorly conserved between different E2. This suggests one mechanism by which the binding of E7 to E2, mediated by the hinge region, may be specific to only certain HPV types. On the E7 protein, the CR3 domain mediates its interaction with E2 through a region spanning residues number 79 to 83. This region of E7 has been shown consist largely of hydrophobic residues which are important for the structural integrity of the E7 protein (Ohlenschlager et al, 2006). However, the use of the 79-83 mutant of E7 did not abolish its binding to a number of other cellular targets (Baldwin et al, 2006; Bernat et al, 2003; Huh et al, 2005) suggesting that this mutant still retains some structural integrity and activities of the wild-type E7 protein. Hydrophobic residues were also shown to mediate protein-protein interactions (Jones & Thornton, 1996). Indeed, the CR3 domain of E7 binds to several cellular targets (see below). The binding of E2 to this region of E7 may therefore be important in regulating the interaction of E7 with several of its cellular targets.

E2 increases the stability of E7

E2 and E7 can modulate each other's activities *in vivo*. The expression of E2 markedly increased the levels of both endogenous and transiently over-expressed E7. Although E2 can act as both transcriptional activator and repressor in a concentration-dependent manner (Steger & Corbach, 1997), we excluded the role of E2's transcriptional activity in increasing the levels of E7 for the following reasons. First, E7's levels were stabilised by E2 even in the absence of an E2 binding site on the E7 promoter as both proteins were over-expressed in U2OS cells from pcDNA expression plasmids. Second, in U2OS cells, as well as in CaSKi cells which contain the viral LCR, the half-life of E7 was increased in the presence of exogenous E2. In

CaSKi cells, this effect was also independent of the HPV mRNA levels, since at the specific concentrations of E2 and incubation time used in this study we observed no major alterations in E6/E7 mRNA levels as determined by RT-PCR. Finally, the levels of the Δ4 mutant of E7, which is defective in binding E2, were not changed upon the introduction of E2, thus providing further evidence that the stability of E7 is increased due to direct protein–protein interaction between the two proteins. E2 may alternatively stabilise E7 by recruiting it to the insoluble fraction of the cell (Figure 11), inhibiting its interaction with the proteasome (Berezutskaya & Bagchi, 1997; Oh et al, 2004a) or by enhancing the phosphorylation of E7 which is an important regulator of its stability (see below).

Inhibition of E7 co-transforming activity in the presence of E2

We also observed an effect of E2 on inhibiting E7's growth-promoting function in primary rodent cells in the absence of any E2-mediated transcriptional modulation. The ability of E7 to cooperate with an activated EJ-*Ras* oncoprotein in the transformation of primary rodent cells depends on its capacity to interfere with the activity of various cell cycle regulatory proteins (Davies et al, 1993; Zerfass et al, 1995). Although binding to and disrupting the function of pRB is considered to be a major oncogenic activity of E7, many studies have shown that this binding is dispensable in some E7-induced phenotypes and is insufficient for E7-induced transformation (Balsitis et al, 2003; Banks et al, 1990; Brokaw et al, 1994; Jewers et al, 1992; Oh et al, 2004b). Here we show that E2 can interfere with both E7-mediated degradation of pRB (Figure 14) and pRB-independent activities of E7, such as inducing centrosome over-duplication (Figure 13) (Duensing & Munger, 2003). Our assays indicate that E2 can regulate E7's functions through two

possible pathways. First, the *in vitro* binding assays showed that E2 binds directly to the C-terminal half of E7 (Figure 7) at a region where several cellular targets of E7 also bind; such as TBP (Massimi et al, 1997), Mi2β-NURD histone deacetylase complex (Brehm et al, 1999) and the AP1 transcription factor (Antinore et al, 1996). Therefore, the binding of E2 to E7 might compete with the binding of E7 to some of its cellular targets and thus inhibit its activities. Secondly, E2 may repress the activity of E7 by changing the localisation of E7 to insoluble compartments within the cell (Figure 11). By doing so, E2 can inhibit the interaction of E7 with several of its cellular partners and possibly redirects E7's activities to other, as yet unidentified, cellular targets. In a normal viral life cycle, the ability of E7 to form a complex with E2 might widen the repertoire of its cellular targets which may be important for a productive viral life cycle. These additional activities of E7 would therefore cease when the expression of E2 is lost as a consequence of viral genome integration.

Reversion of E7-induced centrosome over-duplication by E2

The exact mechanism by which HPV infections induce centrosomal abnormalities is still unknown. The incidence of cells harbouring abnormal centrosome numbers increases with the progression of HPV-induced carcinogenesis (Skyldberg et al, 2001) suggesting that the upregulation of E6 and E7 expression is linked to the severity of centrosome abnormality. Both E6 and E7 were shown to be capable of increasing centrosome numbers in cells (Duensing & Munger, 2002a) where the activities of E7 seems to directly induce centrosome over-duplication (see below). Numeric centrosome abnormalities, however, are also seen in raft cultures harbouring both wild-type HPV-16 genomes as well as genomes mutated in E7

expression (Duensing et al, 2001b) indicating that centrosome over-duplication can occur in early stages of HPV-associated carcinogenesis and possibly as a result of E6 expression alone. Our results are consistent with this observation for the following reasons. We observe that the expression of E2 can inhibit centrosome abnormalities induced by E7, therefore in raft cultures harbouring wild-type HPV genomes, the expression of E2 leads to a phenotype of centrosome numbers that is comparable to raft cultures harbouring mutated HPV genomes with disrupted E7 expression. In addition, the ability of E6 to induce centrosome abnormalities is thought to be an indirect result of its activities, such as degrading p53 (see below), which is not inhibited in the presence of E2 (Grm et al, 2005).

There are various mechanisms by which the expression of E7 can lead to centrosome abnormalities. The activation of cyclins A and E by E7, which are involved in centrosome duplication (Matsumoto et al, 2006; Meraldi et al, 1999), may be one such possibility. It will therefore be interesting to investigate the effects of E2 on this activity of E7, in order to determine which might contribute to its inhibition of E7-induced centrosomal abnormalities. Furthermore, it would be also interesting to investigate the activity of the SCF-ubiquitin ligase complex in the presence of E2; the SCF complex not only binds and regulates the stability of E7 (Oh et al, 2004a), but is also shown to localise to the centrosome (Freed et al, 1999) and its loss of expression results in genomic instability and centrosome over-duplication (Nakayama et al, 2000). E7 may mediate some of its centrosome amplification activities through binding to components of the SCF complex and its eventual destabilisation by the complex may be only an indirect consequence of this interaction. The expression of E2 may not only inhibit SCF-mediated degradation of E7, resulting in its increased stability, but may also inhibit SCF-dependent

centrosome over-duplication mediated by E7. Other unknown cellular targets regulated by E7, which may contribute to E7-induced centrosome abnormalities, may also be targeted by E2. Our results however cannot rule out the possibility that E2 can inhibit E7's activities, in both centrosomal abnormalities and primary cell transformation assays, via additional mechanisms that are independent of its interaction with E7. Indeed, we have also observed that E2 can interfere with some activities of cellular oncoproteins, such as Mdm2, which leads to the rescue of proteasome-mediated degradation of both p53 and pRB (discussed below). We speculate that this activity of E2 does not correlate with its inhibition of E7-mediated centrosome abnormalities since pRB degradation by E7 has been shown to be dispensable for this activity of E7 (Duensing & Munger, 2003) and p53 degradation induced by E6 does not induce centrosome abnormalities in transient transfection assays (discussed further below) (Duensing et al, 2000).

E7 enhances E2's transcriptional activity

E2-induced re-localisation of E7 to the more insoluble fraction of the cell suggests that E2 may recruit E7 onto the chromatin; E2's previously reported localisation (Donaldson et al, 2007; Kurg et al, 2005). We considered that this re-localisation of E7 may be relevant for some of E2's activities and so investigated the effects of E7 on two functions of E2 including transcriptional activation and binding to mitotic chromosomes. The expression of E7 along with E2 in a transient transcriptional activation assay enhances E2's transcriptional activity. We show that the levels of E2 are unchanged in the presence of E7 in this assay, therefore alternative mechanisms by which E7 can enhance this activity of E2 have to be considered. These may include modulating E2 DNA binding affinity or the recruitment of

factors required for optimal E2 transcriptional activity by E7. In the latter case, E7 has been shown to bind to several transcriptional factors including TBP (Massimi et al, 1997) which is also involved in E2-mediated transcriptional activation. Both E2 and TBP bind to the same region on E7. The ability of E7 to dimerise (Zwerschke et al, 1996) may bring both proteins into close proximity by allowing each protein to bind to each of E7 molecule within a dimer. In addition, the binding of E7 to other transcriptional factors, such as AP1 (Antinore et al, 1996), MPP2 (Luscher-Firzlaff et al, 1999), c-myc (Wang et al, 2007) and E2F1 (Hwang et al, 2002), and transcriptional modulators, such as pCAF (Avvakumov et al, 2003) and histone deacetylase (Brehm et al, 1999), may also play a role in mediating E7's enhancement of E2's transcriptional activity. Obviously, these hypotheses need to be further confirmed by, for an example, chromatin-immunoprecipitation to detect E7-mediated changes in the constitution of protein complexes that bind along with E2 on the DNA, as well as to confirm that E7 is actually present at the HPV promoter in the presence of E2.

Localisation of E7 with E2 on mitotic chromosomes

We also found an intriguing co-localisation of E7 with E2 on mitotic chromosomes. The first suggestion that a papillomavirus-encoded protein can associate with mitotic chromosomes came from a study of BPV E2 (Skiadopoulos & McBride, 1998). BPV E2 was shown to bind to the bromodomain-containing protein, Brd4 (You et al, 2004), which attaches to acetylated chromatin during interphase and mitosis (Dey et al, 2003). Although BPV and HPV E2 proteins display identical DNA-sequence specificity and similar functions, they vary in some of their characteristics (Bouvard et al, 1994a; Hines et al, 1998). Here, we have

observed that HPV-16 E2 also possesses a chromosome-association phenotype but, unlike BPV-1 E2, it localises to chromosomes only at late stages of mitosis. This is in agreement with previous studies showing that HPV-16 E2, as well as other alpha papillomaviruses E2 proteins, closely associate with mitotic chromosomes at telophase but not at anaphase or metaphase (McPhillips et al, 2006). However, unlike that study, we did not oberve the chromosomal localisation of HPV-16 E2 at prophase. In addition, the exclusion of HPV-16 E2 from metaphase chromosomes was shown in another study (Van Tine et al, 2004). In contrast to that study, we did not observe a pattern of HPV-16 E2 expression that indicates its localisation to mitotic spindles or centrosomes. These differences might be due to variations in cell lines, expression levels of E2, as well as detection and fixation procedures used. Further studies are required to clarify these issues.

Most interestingly, we observed that 16E2 re-localises E7, but not E6, to mitotic chromosomes at telophase. This suggests that the re-localisation of E7 by E2 is a highly specific event. This was further borne out by the use of an E7 mutation that prevents its binding to E2, which likewise also failed to co-localise with E2 on mitotic chromosomes. At this stage we cannot exclude the possibility that this E7 mutation may have other properties which preclude E2 stabilisation and recruitment to chromosomes, and future studies will aim to clarify this by using E2 mutations that are defective for binding to E7. The biological consequence of this specific localisation is also still unidentified and it remains to be determined whether E2 alters E7 patterns of expression in the context of a normal viral life cycle. This could be investigated using raft culture models of HPV infection. However, these studies are particularly intriguing when one considers the effects of E6 and E7 upon mitosis, where both have been shown to separately induce mitotic

abnormalities when stably expressed in cultured cell-lines or cells derived from transgenic mice (Duensing & Munger, 2002; Patel et al, 2004; Schaeffer et al, 2004). Further dissection of the role of each protein showed that, when each protein is transiently expressed, only E7 results in immediate chromosomal abnormalities (Duensing et al, 2001a; Duensing et al, 2000). This suggests that E7 has a direct effect in inducing centrosomal abnormalities, while the effects induced by E6 might be an indirect consequence of the abrogation of p53 function. Together with the direct role of E7 in interfering with the mitotic machinery, the tethering of E7 to mitotic chromosomes may be required to directly inhibit the cellular checkpoint proteins that might be activated in response to the detection of E2 on mitotic chromosomes, thus avoiding cell cycle arrest in mitosis. Another possible function would be to segregate E7 itself and thus ensure that appropriate amounts of the protein will be present in newly divided daughter cells. E7 might also be required to stabilise the binding of E2 to the viral episomes or mitotic DNA during cell division and current studies are aimed towards assessing the effects of E7 upon E2 during exit from mitosis.

Conclusions: E2-E7 interaction

Overall, these results provide evidence of a novel interplay between the HPV-16 E2 and E7 proteins that may be of relevance during the viral life cycle. In lower basal layers of the epithelium, the localisation of E7 with E2 on mitotic chromosomes may play a role in viral genome segregation which acts to insure the maintenance of viral infection and the progression of viral genomes to upper epithelial layers where the virus completes its life cycle. In this layer, both E2 and E7 are thought to be expressed at low levels, reviewed by (Doorbar, 2005). Further

evidence that both proteins are expressed in this layer, as well as the upper epithelial layers, comes from the analysis of viral transcripts in transgenic mice (Sethi & Palefsky, 2004). The expression of E7 was also shown to overlap with E4 in upper epithelial layers where viral genome amplification and high expression of E2 are expected to take place (Middleton et al, 2003). Overall, these studies suggest that both E2 and E7 can be expressed simultaneously from early to late stages of the viral life cycle and therefore may modulate each other's activities.

E7, as well as E6 (Grm et al, 2005), can enhance E2 transcriptional activity. This enhancement could occur in the lower epithelial layers where the expression of E7 is important for S-phase re-entry of these cells, or in the middle epithelial layer where viral genome amplification takes place. In the latter layer, the expression of E7, and E6, can delay repression of their own transcripts induced by high levels of E2 and therefore prolong viral genome amplification. But whether E7 acts by enhancing E2's transcriptional activation or by delaying transcriptional inhibition remains to be investigated. This enhancement of E2's transcriptional activity by the viral oncoproteins is particularly relevant for the high-risk HPV types which replicate their genomes in upper epithelial layers, in contrast to low-risk HPV types which replicate their genomes in lower epithelial layers where the cellular DNA replication machinery is normally still present (Doorbar, 2005). Our results indicate that E2 and E7 from high-risk HPV types interact at a higher affinity compared to the equivalent proteins derived from low-risk HPV types (Figure 6d), therefore the stimulation of E2 transcriptional activity by E7, as well as E6, may relate to the epithelial layer where viral genome amplification takes place.

In addition, our results show that E2 can inhibit certain activities of E7 which are relevant for cellular transformation and genomic instability. The avoidance of cell transformation in the lower epithelial layer is important to ensure that cellular differentiation is not completely disrupted as a result of E7's activities. By controlling the activities of E7 post-transcriptionally, E2 can maintain the levels of E7 and may act as an ON-and-OFF regulator permitting specific activities of E7 only under certain conditions. The presence of external stimuli (Bosch et al, 2002) may disrupt the E2/E7 interaction stimulating the development of HPV-induced carcinogenesis.

We also show that the expression of E2 inhibited E7-mediated degradation of pRB. In contrast, a previous study characterising the interaction between the E2 and E6 proteins shows that the expression of E2 does not inhibit E6-mediated degradation of p53 (Grm et al, 2005). This indicates that while it is necessary for the virus to circumvent p53-mediated cellular apoptosis at early stages of the viral infection, the activity of pRB, which is also involved in cellular differentiation (Nguyen & McCance, 2005), is finely regulated by the viral proteins. The prevention of apoptosis, by E6, and the induction of cellular differentiation, possibly due to the activities of pRB, are important to ensure the production of mature viral particles prior to their release at upper epithelial layers.

Regulation of E7 by phosphorylation and the proteasome

Identification of a potential Pin1 binding site on E7

Since it was shown that multiple mechanisms were responsible for regulating the function of E7 (Chien et al, 2000; Francis et al, 2000; Oh et al, 2004a), we were also interested in further exploring these aspects. Obviously, CKII phosphorylation has been shown to be critical in this, although it seems likely that there are still numerous gaps in our knowledge of how this is regulated. In this study we provide evidences of the existence of additional phosphorylation events on E7 that have not been described so far. These were initially based on the observation that inhibiting the dephosphorylation of E7 by the PP2A inhibitor, okadaic acid, results in the stabilisation of both wild-type E7 and a mutant of E7 that cannot be phosphorylated by CKII (Figure 19). We therefore analysed the HPV-16 E7 sequence for the presence of consensus sites of other cellular kinases, in addition to CKII, and although there were several potential sites, none of them matched with a high score. We also checked for the consensus site for Pin1, which is upregulated in a number of cancers and whose over-expression is associated with centrosomal abnormalities (Mantovani et al, 2004; Suizu et al, 2006). Pin1 as has also been shown to bind to HBV x-protein (HBx) and increase its stability as well as HBx-mediated transactivation (Pang et al, 2007). HPV-16 E7 contains one potential Pin1 binding site (TP) located at its extreme N-terminal domain (amino acids 5 and 6). Since Pin1 requires the phosphorylation of the threonine or serine residue before binding (Wulf et al, 2005), amino acid number 5 of HPV-16 E7 is expected to be potentially phosphorylated. The binding of Pin1 to E7 increases the level of

E7 and may enhance its oncogenicity and mediate some of its centrosomal abrogation activities.

The activity of CDKs is important for the stability of E7

The Pin1 binding site matches the minimum consensus of a CDK phosphorylation site so we hypothesised that threonine 5 of HPV-16 E7 may be recognised by CDKs for phosphorylation. Here we show that E7 can be phosphorylated *in vitro* by CDK2 and that the activity of CDK2 is important in controlling the stability of E7 *in vivo*. Since E7 has been shown to increase the activity of CDK2 (He et al, 2003) and the expression of cyclin E and cyclin A (Zerfass et al, 1995), it is possible that by doing so, E7 can act positively on increasing its own protein stability. Both HPV-16 and HPV-18 E7 were shown previously to bind to CDK2-containing complexes (He et al, 2003; McIntyre et al, 1996). In the case of HPV-18 E7, its binding to the CDK2/cyclin E complex was shown to be indirect, but mediated by p107 (McIntyre et al, 1996). When we scanned the HPV-18 E7 sequence we found that it contained neither a potential CDK phosphorylation site nor a cyclin binding motif (RXL), therefore indicating that HPV-18 E7 cannot directly interact with cyclins and that this interaction is probably not conserved between E7 proteins from different HPV types. In contrast, HPV-16 E7 contains one potential cyclin binding site which spans amino acids 77-79 as well as a potential CDK2 phosphorylation site at its N-terminal half. The exact residues that are phosphorylated on E7 by CDK2 are still to be determined. Mutants of E7 in both the 5th and 7th threonines are still phosphorylated *in vitro* by CDK2 and are stabilised upon the expression of CDK2 *in vivo*, therefore indicating the possibility of additional CDK phosphorylation sites on E7. This is also supported by the result

that these mutants of E7 still retain their ability to bind Pin1 *in vitro* (data not shown). Furthermore, the binding of the CDK/cyclin complex to HPV-16 E7 by itself may result in its increased stability where the cyclins play an important role in mediating CDK recognition of their substrates. The Δ3 mutant of E7, which contains a disrupted RXL motif, and the N-terminal half fragment, however, are still phosphorylated *in vitro*. It is also possible that CDK2 stabilises E7 indirectly through a third, yet unidentified, proteins which can directly affect the stability of E7.

The precise CDK/cyclin complex that can most efficiently phosphorylate E7 *in vivo* is still to be determined. We observed an increase in the stability of E7 in G1/S-phase of the cell cycle indicating that the CDK2/cyclin E may mediate E7 phosphorylation. However, we cannot exclude the role of other CDK complexes, such as CDK4/cyclin D which are also active at the G1/S-phase (Massague, 2004). At this stage of the cell cycle, the activity of E7 is important in inducing S-phase progression by mediating pRB degradation as well as interfering with initial stages of the centrosome duplication cycle; therefore increasing the stability of E7 through the activity of CDKs may enhance some of E7's activities.

The role of CDK/cyclin complexes in mediating the stability of E7 may be indirectly linked to previously published observations. First, the SCF complex containing the F box proteins, Skp2, which has also been shown to degrade cyclin E (Strohmaier et al, 2001) was shown to result in reduced stability of the E7 protein (Oh et al, 2004a). Second, the use of CDK inhibitors abolished centrosome abnormalities induced by E7 (Duensing et al, 2004). In this case, the authors did not monitor the protein levels of E7 in the presence of the CDK inhibitors. Our

results indicate that the activity of CDK2 is important for the stability of E7, therefore reducing the activity of CDK2, through the expression of the SCF complex or the use of CDK inhibitory molecules, may destabilise E7 and consequently reduce its activity.

CKII phosphorylation of E7 regulates its stability

CKII phosphorylation of E7 was previously shown to enhance its ability to promote S-phase progression (Chien et al, 2000) and cellular transformation (Firzlaff et al, 1991). Here we show that the phosphorylation of E7 by CKII is also important for its stability (Figure 20) suggesting that mutants of E7 that cannot be phosphorylated by CKII may be reduced in their activities as a result of their decreased stability. Analysing the half-life of the CKII mutant of E7 in comparison to the wild-type protein would be a direct way to address this. The stability of E7 appears to be dramatically decreased upon the individual inhibition of either CDK2 or CKII (Figure 20) therefore indicating that the activities of both kinases are not redundant and that inhibiting the activity of one kinase is not compensated by the other. Phosphorylation of E7 by CKII was previously shown to affect its structural conformation (Kee et al, 1998) which may modulate its binding capacity to other proteins. Indeed, CKII phosphorylation was previously shown to enhance complex formation between E7 and TBP (Massimi et al, 1996). These results suggest that phosphorylation of E7, by either CKII or CDK2, may modulate its cellular localisation as well as its ubiquitination and interaction with the proteasome which may therefore affect its stability.

The role of Mdm2 in regulating E2 and E7

Mdm2 accelerates proteasome-mediated degradation of E7

We also propose a third novel mechanism by which the activities of HPV-16 E7 can be controlled. This involves the interaction of E7 with Mdm2 and its consequent degradation by the proteasome. Evidence from a number of experiments support this hypothesis including transient expression assays, RNA-interference, half-life experiments and indirect immunofluorescence. First, inhibiting the expression of Mdm2 using siRNA against Mdm2 results in increased expression levels of E7 when expressed transiently in U2OS cells. Secondly, we observed a prolonged half-life of E7 in Mdm2-/- p53-/- MEF cells (up to 2 hrs) that greatly exceeds the half-life of E7 observed in U2OS cells (between 15 and 30 min) which do not express the Mdm2 inhibitor, p14ARF. The ectopic expression of Mdm2 in Mdm2-/- p53-/- cells resulted in a reduced half-life of E7 that resembles that found in U2OS cells. Finally, the use of proteasome inhibitors rescued Mdm2-mediated degradation of E7, suggesting that the reduced expression of E7 in the presence of Mdm2 involves the proteasome. Overall, these results show that Mdm2 enhances the proteasome-mediated degradation of E7, although whether this is ubiquitin-dependent or ubiquitin-independent is still to be determined. Immunofluorescence analyses, however, show that, in the presence of proteasome inhibitors, E7 localises with Mdm2 into nuclear structures that are enriched with ubiquitinated proteins. This may indicate that Mdm2 enhances ubiquitin-dependent proteasome degradation of E7 and is consistent with previous results showing that E7 is indeed poly-ubiquitinated *in vivo* (Oh et al, 2004a; Wang et al, 2001). Furthermore, these nuclear structures co-localise with PML bodies

suggesting that the co-localisation of E7 to PML bodies (Bischof et al, 2005) may mediate its degradation. This is also seen in CaSKi cells where, upon the inhibition of the proteasome, both E7 and PML appear to co-localise (Oh et al, 2004a). The destabilisation of E7 by Mdm2 may be an indirect consequence of its localisation to PML bodies where it acts to inhibit PML IV-induced senescence by disrupting pRB and p53/CBP activities (Bischof et al, 2005). This specific localisation of E7 to nuclear structures, which both mediates some of its functions and controls its activities, may be a mechanism by which E7 can be finely regulated.

Our results provide the first evidence that Mdm2 can mediate the degradation of a viral gene product. The expression of E7 was shown to induce p53 levels which results in an increased expression of Mdm2 (Thomas & Laimins, 1998). This may lead to a negative feedback loop whereby the cell can oppose increased levels of E7. However, the expression of E6 (which targets p53 for degradation) along with E7 was shown to inhibit this increased expression of Mdm2 (Thomas & Laimins, 1998). Therefore, the expression of full-length E6 protein, along with E7, during the viral life cycle may play an indirect role in inhibiting Mdm2-mediated degradation of E7. Interestingly, it was recently shown that p19ARF (mouse homologue of Human p14ARF) induces the relocalisation of E7 to the nucleolus and also inhibits some of E7's activities (Pan et al, 2003). This is mediated through two sites on p19ARF that are important for mediating the nucleolar localisation of Mdm2 (Weber et al, 2000). In their assays, Pan, *et al*, could not detect any binding between p19ARF and E7. Therefore, the ARF protein-mediated localisation of Mdm2 to nucleoli may lead to the relocalisation of E7 as a result of E7's binding to Mdm2. Our results however indicate that the activity of the ARF protein is dispensable for the localisation of Mdm2 (Bernardi et al, 2004), as well as E7, to the

nucleolus, since Mdm2-E7 localisation at nucleoli is observed in U2OS cells which are mutated in p14ARF expression.

Mdm2 is the second ubiquitin ligase identified that acts to destabilise E7. The SCF complex was previously shown to decrease the half-life of E7 and mediate its proteasome degradation (Oh et al, 2004a). E7 may be differentially targeted for degradation by either ubiquitin ligase complex depending on the stage of the cell cycle or the stimulation of certain pathways. The SCF complex targets multiple cell cycle regulatory proteins most of which are phosphorylated by CDKs before their degradation (Spruck & Strohmaier, 2002) indicating that the degradation of E7 by the SCF complex may depend on its phosphorylation status. The ability of E2 and CDK2 to increase the stability of E7 may occur through inhibiting the interaction of E7 with the SCF complex since we saw no rescue of Mdm2-mediated degradation of E7 upon increased expression of E2 or CDK2 (Figure 24). Therefore, the increase in E7 levels in G1/S-phase may be due to the inhibition of the SCF binding to CDK2-phosphorylated E7. Little is known about the regulation of Mdm2's activities during the cell cycle in comparison to what is known about its stimuli-induced regulation (Meek & Knippschild, 2003). Almost 20% of the Mdm2 protein sequence is composed of serine and threonine residues suggesting that phosphorylation plays an important role in regulating its activities. DNA damage-induced kinases such as DNA-activated protein kinase (DNA-PK) and ATM were shown to phosphorylate Mdm2 and impede its ability to degrade p53 (Khosravi et al, 1999; Mayo et al, 1997). In this case, inhibiting Mdm2-mediated degradation of E7 may be important in order to circumvent restriction of cellular growth as a response to DNA damage. Furthermore the PI3-K/PKB pathway, which promotes cell proliferation stimulated by growth factors and cytokines, was also shown to

increase the phosphorylation of Mdm2 and enhance its localisation to the nucleus (Mayo & Donner, 2001). Here, the ability of E7 to induce cellular proliferation can be substituted for by normal cellular pathways, and therefore its possible destabilisation by Mdm2 may be affordable for the virus.

Interaction between E2 and Mdm2

Several lines of evidence indicate a pro-apoptotic activity of E2 where taken out of the context of HPV gene expression. For example, papillomavirus E2 can induce apoptosis in a manner independent of other viral protein activities (Demeret et al, 2003; Webster et al, 2000) and the expression of the N-terminal half of E2 (lacking the DNA binding domain) results in growth inhibition of HPV-positive cell lines (Desaintes et al, 1999). In addition, full-length E2 stimulates p53 transcriptional activity in HPV-negative cell lines (Desaintes et al, 1997) and the expression of a dominant-negative p53 reduced apoptosis mediated by HPV-16 E2, but not HPV-18 E2 (Desaintes et al, 1997; Webster et al, 2000). Here, we propose a novel mechanism by which HPV-16 E2 can regulate p53 activity by binding to Mdm2 through its C-terminal domain, but an additional role of the N-terminal domain cannot be ruled out. We detected a significant binding between Mdm2 and E2 both *in vitro* and *in vivo*. Although a similar region on the E2 protein mediates its interaction with p53 and Mdm2, we propose that the binding between E2 and Mdm2 is independent of E2's binding to p53 for two reasons. First, E2 inhibits Mdm2-mediated degradation of pRB in a p53 negative background (Figure 28). Second, E2 and Mdm2 co-localise in SAOS-2 and Mdm2-/- p53-/- cells (Figure 30c) in the presence of proteasome inhibitors. These structures resemble in their appearance and constitution nuclear stress bodies that form in the presence of

cellular stress, such as the inhibition of the proteasome. Our results indicate that E2 can markedly inhibit Mdm2-mediated degradation of pRB and p53. In both cases, further investigation is required to show whether both proteins still retain their cellular activities in the presence of E2. In an HPV infection, the retention of p53 activity may be important at later stages of the life cycle at the upper epithelial layers. In this stage, the expression of Mdm2 and p53 may increase as a result of reduced E6 expression. E2 may therefore stimulate p53-mediated apoptosis to aid virion release by inhibiting Mdm2-mediated degradation of p53 (Blachon & Demeret, 2003).

We did not detect any change in the levels of E2 in the presence of Mdm2, but we observed that Mdm2 results in an increased transcriptional activation by E2. This is independent of pRB and p53 degradation by Mdm2 as this phenomenon is observed in both pRB/p53-positive and –negative cell lines. The role of the ubiquitin-proteasome machinery in transcription has been recently highlighted (Muratani & Tansey, 2003). One example of this is the regulation of c-myc transcriptional activities through its ubiquitination by the HectH9 ubiquitin-ligase (Adhikary et al, 2005). Ubiquitination of c-myc appears to be important for the recruitment of the p300 co-activator which is important for transcriptional activation of c-myc-regulated genes. In another case, the proteasome machinery was shown to affect oestrogen receptor α (ERα) transcriptional activity (Reid et al, 2003). Here, the proteasome components were shown to bind to the promoter region and are important for the cycling of ERα on the transcription complex. Finally, in an HPV-related model, the activation of hTERT expression by HPV-16 E6 was shown to be dependent on the interaction between E6 and E6AP, where both proteins were shown to bind interdependently to the hTERT gene promoter

(Liu et al, 2005). In the case of Mdm2, its expression was shown to increase the transcriptional activity, by a poorly described mechanism, of a number of factors such as the p53 homolog p63 (Calabro et al, 2002) and ERα (Saji et al, 2001). Here, we show that Mdm2 can also increase the transcriptional activity of HPV-16 E2. Further investigation is required to identify whether Mdm2 is required to increase the ubiquitination of E2 by Mdm2, modulate the recruitment of cellular factors which may facilitate E2's transcriptional activity or affect the binding of E2 to DNA. Furthermore, the localisation of E2 to PML bodies by Mdm2 may well aid the transcriptional activity of E2. Many transcription factors seem to congregate in nuclear structures such as PML bodies (Borden, 2002) which is thought to aid their activities. PML bodies were shown to enhance papillomavirus infectivity and transcription (Day et al, 2004) and this correlates with an increase in the efficiency of viral infection of a number of DNA tumour viruses (Everett, 2001). Mdm2 may increase viral gene expression from the late viral promoter resulting in an increased expression of the *E4*, *L1* and *L2* gene products and therefore facilitating virion production. Furthermore, localisation of E2 to PML bodies was shown previously to be mediated by L2 which is thought to enhance viral genome packaging (Day et al, 1998). Therefore, Mdm2 might also act to facilitate viral genome packaging by enhancing the localisation of E2, and in turn viral DNA, to PML bodies. Overall, these results indicate a novel cross-talk between the viral transcriptional activator and Mdm2 (Figure 33b).

Materials and Methods

Cells and transfection. U2OS (human osteosarcoma, p53+/+ pRB+/+), SAOS-2 (human osteosarcoma, p53-/- pRB-/-), Baby Rat Kidney (BRK), Mdm2-/- p53-/- mouse embryo fibroblasts (MEF), 293 (human embryonic kidney) and CaSKi (human cervical carcinoma, HPV16 positive) cells were grown in DMEM supplemented with 10% fetal bovine serum, penicillin-streptomyocin (100U/ml) and glutamine 292μg/ml. Transfection was carried out using calcium phosphate precipitation as described previously (Matlashewski et al, 1987) or using Lipofectamine2000 (Invitrogen) according the manufacturer's protocol.

Plasmids.

E2: Expression plasmids encoding GST-tagged 16E2 were described previously including full-length GST-16E2 (Piccini et al, 1995) and N- and C-terminal halves (Massimi et al, 1999). C-terminal deletion mutants spanning residues 249-365 were amplified by PCR using the following primers: forward primer 5' GACACCGGATCCCCCTGCCACACC; reverse primer 5' GTTGTGAATTCAGTATCAAGATTTGTCATATA followed by digestion using BamHI and EcoRI restriction enzymes and cloning into pGEX-2T plasmid. 16E2 in pcDNA was described previously (Bouvard et al, 1994a). GST-11E2 was generated by PCR amplification of a GFP-tagged HPV-11E2 construct (Merilyn Hibma) using the following primers: 5'-AGCGGATCCATGGAAGCAATA-3' (F), 5'-AGCGAATTCTTACAATAAATGTAATGA-3' (R) and cloned into pGEX-2T plasmid using BamHI/EcoRI restriction sites. All Constructs were verified by

DNA sequencing. The N- and C- terminal halves of 16E2 used for *in vitro* translation-transcription were cloned into pSP64 plasmid as described previously (Piccini et al, 1995). For *in vivo* expression the following plasmids encoding 16E2 were used CMV.16E2 (Bouvard et al, 1994a) and GFP-16E2 kindly provided by Ian Morgan.

E7: For bacterial expression of 16E7, plasmids encoding full-length GST-16E7 (Massimi et al, 1996) and His-tagged 16E7 (Prathapam et al, 2001) were described previously. The N- and C-terminal halves of E7 were cloned into pGEX-2T using BamHI/EcoRI restriction sites by PCR amplification using the following primers, for the N-terminal half of 16E7: forward primer 5' ACGTAGGGATCCCCAGCTGTAATC; reverse primer 5' CTGGAATTCCAGCTGGACCATCTAT and for the C-terminal half of 16E7: forward primer 5' CCAGGATCCCAAGCAGAACCGGAC; reverse primer 5' CTCTTCCGAATTCGTACCTGCAGG. For *in vitro* translation transcription of E7 the following plasmids were used; pSP64.16E7 (Massimi et al, 1996); pSP64 encoding the C-terminal mutants of 16E7 (Massimi et al, 1996) and 11E7 cloned into pcDNA from pJ4Ω plasmid (Storey et al, 1988) using EcoRI and HindIII restriction sites. For *in vivo* expression of 16E7 the following plasmids, described previously, were used; pJ4Ω.16E7 (Storey et al, 1988), CKII mutant of 16E7 (pcDNA.16E7 31/32) (Edmonds & Vousden, 1989) and N-terminally tagged HA-16E7 (Guccione et al, 2002). Untagged E7 was cloned into pcDNA from pJ4Ω.E7 using BamH1/HindIII restriction sites and the Δ3 and Δ4 mutants of E7 were cloned from pSP64 plasmid into pcDNA using EcoR1/BamH1 restriction sites. Subsequently, point mutants of E7 (including T5A, T57A, S71 and S71T57A) were

mutated into alanine residues using site directed mutagenesis and verified using DNA sequencing.

Additional *in vitro* expression plasmids. For bacterial expression of GST-tagged proteins the following constructs were used; GST-16E1 (Storey et al, 1995); GST-TBP (Massimi et al, 1997).and GST-p53 (Massimi et al, 1999). For *in vitro* translation-transcription of Mdm2, a pSP65.Mdm2 was cloned by Miranda Thomas using EcoRI restriction sites.

Additional *in vivo* expression plasmids. The following plasmids used were previously described as follows; pGFP-N1 (Clontech), pGFP-NLS (Clontech), Adenovirus E1a (Matlashewski et al, 1987), EJ-*Ras* (pEJ6.6) (Storey & Banks, 1993), p6xE2BS-Luc reporter plasmid was kindly provided by Ian Morgan; CMV.pRB, pcDNA Pin1 and GST-Pin1 were kindly provided by Giannino Del Sal, pcDNA3 HA PP2A Cα expressing the 35 kDa catalytic subunit of PP2A and PP2A dominant negative (DN) (Pim et al, 2005), cyclin E and HA-tagged CDK2 expression constructs were kindly provided by Ole Gjoerup, FLAG-p53 was kindly provided by Georgine Faulkner and Mdm2 pCoC expression plasmid was kindly provided by Karen Vousden.

Antibodies. Mouse monoclonal antibody against E2 (IF 1:100) and anti-E2 polyclonal antibody (IF 1:200, WB 1:5000) have been described previously (Hibma et al, 1995; Massimi et al, 1999)

The following commercial antibodies were used at the dilutions indicated in brackets: anti-HA monoclonal antibody 12CA5 (Roche; WB 1:100, IF 1:100) anti-β-galactosidase β-gal (Promega, WB 1:5000), polyclonal rabbit anti-α-actin (Sigma

1:1000), mouse anti-16E7 ED17 (Santa-Cruz, also kindly provided by K. Münger, WB 1:100, IF 1:100) polyclonal rabbit anti-HA Y-11 (Santa-Cruz, IF 1:50), anti-PML rabbit polyclonal antibody (Santa Cruz, IF 1:100), anti-Daxx rabbit polyclonal antibody M-112 (Santa Cruz, IF 1:100), anti-Flag mouse monoclonal antibody M2 (Sigma, WB 1:1000, IF 1:1000), anti-Flag rabbit polyclonal antibody (Sigma, IF 1:1000), anti-GFP polyclonal antibody (Santa Cruz, WB 1:1000), mouse anti-p53 DO-1 (Santa Cruz, WB 1:1000, IF 1:100), goat anti-nucleolin C23 (Santa Cruz, IF 1:50), mouse anti-pRB (WB 1:500), mouse anti-γ-tubulin (Sigma; WB 1:5000, IF 1:200), mouse anti-conjugated ubiquitin FK2 (Biomol, 1mg/ml stock, IF 1:500), mouse anti-Mdm2 (Calbiochem, IF 1:30; also the mouse 2A10 antibody kindly provided by Giannino Del Sal, WB: 1:100).

Appropriate secondary antibodies conjugated to HRP were purchased from DAKO and used for Western blotting at a dilution of 1:2000. In the case of immunofluorescence staining, secondary antibodies conjugated to either fluorescein or rhodamine were purchased from Molecular Probes and used at a concentration of 1:700.

BRK transformation assay. Primary Baby Rat Kidney (BRK) cells from 9-day-old Wistar rats were transfected with either pJ4Ω:HPV-16E7 (6μg), pcDNA 16E7 (3μg) or Adenovirus E1a encoding plasmid (3 or 6 μg) along with 3μg EJ-*Ras* and 3μg pcDNA encoding neomyocin resistance, with or without the indicated amounts of CMV.16E2 or GFP 16E2 expression plasmids. Cells were placed under selection in growth medium containing 200μg/ml G418 for 2 weeks and then fixed, and

colonies were stained with Giemsa-Blue (Diagnostica Merck) and then counted. The results obtained are representative of at least 3 independent experiments.

Fusion protein purification and *in vitro* binding assays. GST- and His-tagged fusion proteins were expressed and purified as described previously (Thomas et al, 1996). Briefly, 40ml of an overnight culture of *E. coli* strain DH5-α previously transformed with the appropriate expression plasmids were inoculated in a one to ten volume of Luria Broth (LB) containing ampicillin and grown at 37°C up to an OD of 0.6 at 395nm. Recombinant protein expression was induced for 3 hrs with 1nM isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma). The cells were harvested by centrifugation, disrupted by sonication in lysis buffer (PBS, 1% Triton X-100, 100 U/ml DNAse, protease inhibitors cocktail, Calbiochem) and the lysates were then cleared from cell debris by centrifugation. The GST-fusion proteins were then incubated for 1 hr with glutathione-conjugated agarose. In the case of Histidine protein purification, the following lysis buffer was used (10mM Tris-HCl pH8.8, 300mM NaCl, 0.1 M phosphate buffer pH 8.0, 100U/ml DNAse) and Ni-NTA (Qiagen Inc.) beads. His-fusion proteins were eluted with increasing amounts of imidazole. The purity of all fusion proteins was determined by SDS-PAGE and Coomassie Brilliant Blue R (Sigma) staining.

In vitro translation transcription was performed in rabbit reticulocyte lysate or wheat germ extract using the Promega TNT system and were radiolabelled with [^{35}S]cysteine (Amersham). Equal amounts of *in vitro*-translated proteins were added to GST fusion proteins bound to glutathione resin and incubated for 1 hr at 4°C. After extensive washing with PBS containing 0.25% NP-40, or as otherwise indicated, the bound proteins were analysed by SDS-PAGE and autoradiography.

Binding assays were quantified using PhosphorImager and the percentage binding with respect to inputs was calculated.

GST pull downs from cellular extracts was performed by incubating GST-fusion proteins immobilised on resin with cell extract for 1 hr at 4°C on a rotating wheel. The resin was then washed extensively with the extraction buffer and bound proteins were detected using SDS-PAGE and Western blot using the appropriate antibodies.

Direct binding assays were performed by incubating GST fusion proteins immobilised on resin with purified His-tagged 16E7 that had been eluted with imidazole containing buffer, for 1h at 4°C. After extensive washing the bound proteins were analysed by SDS-PAGE and Western blotting using anti-16E7 antibodies.

Immunoprecipitation and Western blotting. Total cellular extracts were prepared by directly lysing cells from 6-well dishes in SDS loading buffer. To obtain the soluble and insoluble cellular fractions separately, cells were lysed in either low-salt E1A buffer (25mM HEPES pH 7.0, 0.1%NP-40, 150mM NaCl, plus protease inhibitor cocktail I; Calbiochem) or high-salt E1A buffer containing 500mM NaCl. After incubation on ice for 20 min lysates were cleared by centrifugation at 1300 rpm for 10min. The supernatant (soluble fraction) and pellet (insoluble fraction) were analysed by SDS-PAGE and Western blotting. For Western blotting, 0.45 μm nitrocellulose membrane (Schleicher & Schuell) were used and membranes were blocked for 1 hr at 37°C in 10% milk followed by the incubation with the appropriate primary antibody diluted in 10% milk/0.5%Tween

20 for 2 hrs. After several washings with PBS 0.5%Tween 20, secondary antibodies conjugated with HRP (Molecular Probes) were diluted in 1%milk/0.5%Tween 20 and incubated for 1 hr. Blots were developed using Amersham ECL technique according to the manufacturer's instructions. In the case of Western blotting of E7, 0.22 μm membranes were used and membranes were treated with 0.5% glutaraldehyde in TBS for 0.5 hr at room temperature before blocking in 1%milk/TBS for 1 hr at room temperature. Blots were then incubated with monoclonal anti-E7 antibodies (1:100) diluted in TBS 1%milk/1%Tween 20 overnight at 4°C. After incubation in appropriate 2° antibody, the blots were then developed using Femto solutions (Pierce) according to the manufacturer's instructions.

For co-immunoprecipitation, U2OS or 293 cells were transfected with the appropriate plasmids. 24 hrs later, E1A extraction was performed and the soluble fraction was incubated with anti-HA beads (Sigma) to pull down E7, or with anti-GFP antibodies to pull down GFP-E2 for 3-4 hrs on a rotating wheel at 4°C. In the case of GFP immunoprecipitation, Protein A agarose (Amersham) was added to the reaction 40 min prior to final washings. The agarose beads were extensively washed and precipitated proteins were analysed by Western blotting using the antibodies indicated.

Half-life experiments. Lipofectamine2000 was used to transfect CaSKi cells with 3μg of plasmid expressing E2 and Mdm2-/- p53-/- MEF cells with 1μg pcDNA E7 with or without 2μg Mdm2 expression plasmid. 24 hrs after transfection, cells were treated for different time points as indicated with cycloheximide (50μg/ml in

DMSO) to block protein synthesis. Total cellular extracts were then analysed by Western blot and the intensity of the bands on the X-ray film was measured using Adobe Photoshop. The standard deviation was calculated from three independent assays.

RT-PCR. Total RNA was isolated from CaSKi cells (transfected as above) and BRK cells (transfected as in the transformation assay) 24 hrs after transfection using TRI Reagent (Sigma) according to the manufacturer's instructions. A total of 1μg of RNA was subjected to reverse transcription (RT) using the RETROscript system (Ambion). No reverse transcriptase control was also added for assaying contamination with DNA. PCR was performed with 20 cycles and an annealing temperature of 55°C for E2, GFP and actin and 58°C for E7. PCR primers for actin, E7 (Yoshinouchi et al, 2003) and GFP (Grm et al, 2005) have been described previously. 16E2 primers were as follows: forward 5' ATGGAGACTCTTTGCCAA; reverse 5' TCATATAGACATAAATCCAGTAGACAC.

Immunofluorescence and Microscopy. Cells were stained and fixed for immunofluorescence as described previously (Grm et al, 2005). Briefly, cells were fixed with 3.7% paraformaldehyde in PBS for 20min and permeabilised with 0.1% Triton X-100 in PBS for 5min. Primary antibodies were incubated for 2 hr at 37°C, followed by extensive washing in PBS and incubation for 30 min at 37°C with secondary anti-rabbit or anti-mouse conjugated with fluorescein- or rhodamine (Molecular Probes). For the visualisation of chromosomes, cells were stained with

1μM Hoechst (Sigma No. 33258; bisBenzimide). Samples were then washed several times with water and mounted using Vectashield mounting medium (Vector Laboratories Inc.) on glass slides. The results obtained are representative of at least 3 independent experiments.

Slides were analysed using either a Leica DMLB fluorescence microscope equipped with a Leica photo camera (A01M871016) or a Zeiss LSM 510 confocal microscope and the data were collected utilising the X100 objective oil immersion lens. To avoid cross talk between channels during confocal scanning the following settings were used: FITC was excited with a 488nm line of an Argon laser at which the excitation of rhodamine is negligible; rhodamine was excited with a 543nm line of a Neon laser at which the excitation of FITC is negligible. The FITC emission was monitored using a 505-530nm filter and the rhodamine was monitored using a 560nm longpath filter.

Pre-permeabilisation assay. U2OS cells were grown on coverslips in either 6 well dishes or in 6cm dishes and transfected with the plasmids indicated. 24 hrs after transfection, cells were washed with PBS and incubated for 7-min in 0.5% Triton X-100 in 20mM HEPES-KOH (pH 7.9), 50mM NaCl, 3mM $MgCl_2$ and 300mM sucrose (Araujo et al, 2005). Cells were then processed for either immunofluorescence staining or Western blotting (as described above).

Centrosome abnormality assay. U2OS cells were grown on coverslips in 6well dishes. 24 hrs after transfection, cells were treated with nocodazole for a further 24 hrs, and then fixed and processed for immunofluorescence (as described above).

The following antibodies were used; monoclonal anti-γ-tubulin to detect centrosomes, polyclonal anti-16E2 and polyclonal anti-HA to detect co-transfected HA-tagged E7.

Dual Luciferase reporter assay. U2OS and SAOS-2 cells were grown in 6 well dishes and transfected using calcium phosphate and Lipofectamine2000, respectively. Transfected plasmids include, 6xE2BS-Luc (1μg), *Renilla Luciferase* (pRL) (0.01μg) and a combination of GFP-16E2 (1.5μg), pcDNA E7 (as indicated) and Mdm2 (3μg). All transfections were repeated at least three times. Simultaneous expression of the pRL plasmid provided an internal control of baseline response and allowed for the normalisation of transfection efficiency. 24 hrs post transfection, cells were lysed and luciferase activity was measure using the Dual-luciferase assay kit as recommended by the manufacturer (Promega).

Cell cycle synchronisation. To synchronise U2OS cells, aphidicolin (Sigma) was added at a concentration of 4μg/ml to asynchronous growing cells for 24 hrs. The aphidicolin-containing medium was then removed and the cell culture was washed with 10ml of PBS. The PBS was then replaced with complete medium and the cell synchronization was checked by FACS analysis. Cells were harvested at different times (including time point 0 for G1 phase) and DNA content was assessed by propidium iodide staining and FACS analysis as described previously (Banks et al., 1990).

***In vivo* degradation assays.** U2OS (for FLAG-p53 degradation) and SAOS-2 (for pRB degradation) were transfected with 2μg of FLAG-p53 or pRB expression plasmids, along with 0.15μg of LacZ or 3μg of GFP-NLS expression plasmids as indicated. In addition, the following plasmids were also included; CMV.16E2 (3μg), pcDNA E7 (4μg), Mdm2 (4μg). 24 hrs post-transfection, or as otherwise indicated, the cells were harvested and analysed by Western blotting.

Inhibitors. The following inhibitors, summarised in Table 4, were dissolved in DMSO and used at the indicated concentrations: nocodazole (Sigma, 2μM), aphidicolin (Sigma, 5μM), okadaic acid (OA, Sigma, 100nM), roscovitine (Calbiochem, 60μM), apigenin (Sigma, 30μM), CBZ (MG132, Sigma, 50μM), LLnL (Sigma, 50μM), epoxymocin (Sigma, 25μM), Aurora kinas inhibitor II (Calbiochem, 25μM), PIK3 inhibitor (Ly294002, Celbio). In addition, cells treated with DMSO alone were also included as a negative control.

Table 4: Description of the various inhibitors used.

Inhibitor	Nocodazole	aphidicolin	Okadaic acid	Roscovitine	Apigenin
Inhibited molecule	Microtubule	DNA polymerase ε	PP2A	CDK1/CDK2	CKII
Conc.	2μM	5μM	100nM	60μM	30μM
Company	Sigma	Sigma	Sigma	Calbiochem	Sigma

Inhibitors cont.

Inhibitor	Proteasome	Aurora II inhibitor	PIK3 inhibitor	Epoxomycin
Inhibited molecule		AuroraII	PIK3	Proteasome
Conc.	CBZ: 50μM LLnL: 50μM	2.5uM	25uM	25uM
Company	Sigma	Calbiochem	Celbio	Sigma

***In vitro* phosphorylation.** Purified GST fusion proteins were incubated with commercially purified CDK2 kinase (New England Biolabs) for 20 min at 30°C in phosphorylation buffer (20mM HEPES, pH 7.5, 20mM MgCl2, 0.3mM aprotinin, 1 mM/Pepstatin) supplemented with 56nM [32P]ATP (Amersham) and 10mM ATP. After extensive washing, the phosphorylated proteins were monitored by SDS-PAGE and autoradiography.

siRNA experiments. U2OS cells seeded in 6cm dishes and transfected using Lipofectamine2000 (Invitrogen) with pcDNA 16E7 along with control siRNA against luciferase or siRNA against Mdm2 (siMdm2) (Dharmacon) according to the manufacturer's procedure. 48 hrs after transfection, cells were harvested and protein levels assessed by Western blot.

References

Abbate EA, Voitenleitner C, Botchan MR (2006) Structure of the papillomavirus DNA-tethering complex E2:Brd4 and a peptide that ablates HPV chromosomal association. *Molecular cell* **24:** 877-889

Adhikary S, Marinoni F, Hock A, Hulleman E, Popov N, Beier R, Bernard S, Quarto M, Capra M, Goettig S, Kogel U, Scheffner M, Helin K, Eilers M (2005) The ubiquitin ligase HectH9 regulates transcriptional activation by Myc and is essential for tumor cell proliferation. *Cell* **123:** 409-421

Androphy EJ, Lowy DR, Schiller JT (1987) Bovine papillomavirus E2 trans-activating gene product binds to specific sites in papillomavirus DNA. *Nature* **325:** 70-73

Antinore MJ, Birrer MJ, Patel D, Nader L, McCance DJ (1996) The human papillomavirus type 16 E7 gene product interacts with and trans-activates the AP1 family of transcription factors. *Embo J* **15:** 1950-1960

Araujo FD, Stracker TH, Carson CT, Lee DV, Weitzman MD (2005) Adenovirus type 5 E4orf3 protein targets the Mre11 complex to cytoplasmic aggresomes. *J Virol* **79:** 11382-11391

Avvakumov N, Torchia J, Mymryk JS (2003) Interaction of the HPV E7 proteins with the pCAF acetyltransferase. *Oncogene* **22:** 3833-3841

Bagasra O (2005) RNAi as an antiviral therapy. *Expert opinion on biological therapy* **5:** 1463-1474

Baldwin A, Huh KW, Munger K (2006) Human papillomavirus E7 oncoprotein dysregulates steroid receptor coactivator 1 localization and function. *J Virol* **80:** 6669-6677

Balsitis S, Dick F, Lee D, Farrell L, Hyde RK, Griep AE, Dyson N, Lambert PF (2005) Examination of the pRb-dependent and pRb-independent functions of E7 in vivo. *J Virol* **79:** 11392-11402

Balsitis SJ, Sage J, Duensing S, Munger K, Jacks T, Lambert PF (2003) Recapitulation of the effects of the human papillomavirus type 16 E7 oncogene on mouse epithelium by somatic Rb deletion and detection of pRb-independent effects of E7 in vivo. *Mol Cell Biol* **23:** 9094-9103

Banks L, Edmonds C, Vousden KH (1990) Ability of the HPV16 E7 protein to bind RB and induce DNA synthesis is not sufficient for efficient transforming activity in NIH3T3 cells. *Oncogene* **5:** 1383-1389

Barbera AJ, Chodaparambil JV, Kelley-Clarke B, Joukov V, Walter JC, Luger K, Kaye KM (2006) The nucleosomal surface as a docking station for Kaposi's sarcoma herpesvirus LANA. *Science* **311:** 856-861

Barbosa MS, Edmonds C, Fisher C, Schiller JT, Lowy DR, Vousden KH (1990) The region of the HPV E7 oncoprotein homologous to adenovirus E1a and Sv40 large T antigen contains separate domains for Rb binding and casein kinase II phosphorylation. *Embo J* **9:** 153-160

Barbosa MS, Vass WC, Lowy DR, Schiller JT (1991) In vitro biological activities of the E6 and E7 genes vary among human papillomaviruses of different oncogenic potential. *J Virol* **65:** 292-298

Bedrosian CL, Bastia D (1990) The DNA-binding domain of HPV-16 E2 protein interaction with the viral enhancer: protein-induced DNA bending and role of the nonconserved core sequence in binding site affinity. *Virology* **174:** 557-575

Bellanger S, Demeret C, Goyat S, Thierry F (2001) Stability of the human papillomavirus type 18 E2 protein is regulated by a proteasome degradation pathway through its amino-terminal transactivation domain. *J Virol* **75:** 7244-7251

Berezutskaya E, Bagchi S (1997) The human papillomavirus E7 oncoprotein functionally interacts with the S4 subunit of the 26 S proteasome. *J Biol Chem* **272:** 30135-30140

Bernardi R, Scaglioni PP, Bergmann S, Horn HF, Vousden KH, Pandolfi PP (2004) PML regulates p53 stability by sequestering Mdm2 to the nucleolus. *Nature cell biology* **6:** 665-672

Bernat A, Avvakumov N, Mymryk JS, Banks L (2003) Interaction between the HPV E7 oncoprotein and the transcriptional coactivator p300. *Oncogene* **22:** 7871-7881

Bernat A, Massimi P, Banks L (2002) Complementation of a p300/CBP defective-binding mutant of adenovirus E1a by human papillomavirus E6 proteins. *J Gen Virol* **83:** 829-833

Blachon S, Bellanger S, Demeret C, Thierry F (2005) Nucleo-cytoplasmic shuttling of high risk human Papillomavirus E2 proteins induces apoptosis. *J Biol Chem* **280:** 36088-36098

Blachon S, Demeret C (2003) The regulatory E2 proteins of human genital papillomaviruses are pro-apoptotic. *Biochimie* **85:** 813-819

Borden KL (2002) Pondering the promyelocytic leukemia protein (PML) puzzle: possible functions for PML nuclear bodies. *Mol Cell Biol* **22:** 5259-5269

Bosch FX, Lorincz A, Munoz N, Meijer CJ, Shah KV (2002) The causal relation between human papillomavirus and cervical cancer. *Journal of clinical pathology* **55:** 244-265

Bosch FX, Qiao YL, Castellsague X (2006) CHAPTER 2 The epidemiology of human papillomavirus infection and its association with cervical cancer. *International journal of gynaecology and obstetrics: the official organ of the International Federation of Gynaecology and Obstetrics* **94 Suppl 1:** S8-S21

Bouvard V, Matlashewski G, Gu ZM, Storey A, Banks L (1994a) The human papillomavirus type 16 E5 gene cooperates with the E7 gene to stimulate proliferation of primary cells and increases viral gene expression. *Virology* **203:** 73-80

Bouvard V, Storey A, Pim D, Banks L (1994b) Characterization of the human papillomavirus E2 protein: evidence of trans-activation and trans-repression in cervical keratinocytes. *Embo J* **13:** 5451-5459

Boyer SN, Wazer DE, Band V (1996) E7 protein of human papilloma virus-16 induces degradation of retinoblastoma protein through the ubiquitin-proteasome pathway. *Cancer Res* **56:** 4620-4624

Brehm A, Nielsen SJ, Miska EA, McCance DJ, Reid JL, Bannister AJ, Kouzarides T (1999) The E7 oncoprotein associates with Mi2 and histone deacetylase activity to promote cell growth. *Embo J* **18:** 2449-2458

Brokaw JL, Yee CL, Munger K (1994) A mutational analysis of the amino terminal domain of the human papillomavirus type 16 E7 oncoprotein. *Virology* **205:** 603-607

Burgers WA, Blanchon L, Pradhan S, de Launoit Y, Kouzarides T, Fuks F (2007) Viral oncoproteins target the DNA methyltransferases. *Oncogene* **26:** 1650-1655

Calabro V, Mansueto G, Parisi T, Vivo M, Calogero RA, La Mantia G (2002) The human MDM2 oncoprotein increases the transcriptional activity and the protein level of the p53 homolog p63. *J Biol Chem* **277:** 2674-2681

Calderwood MA, Hall KT, Matthews DA, Whitehouse A (2004) The herpesvirus saimiri ORF73 gene product interacts with host-cell mitotic chromosomes and self-associates via its C terminus. *J Gen Virol* **85:** 147-153

Chellappan S, Kraus VB, Kroger B, Munger K, Howley PM, Phelps WC, Nevins JR (1992) Adenovirus E1A, simian virus 40 tumor antigen, and human papillomavirus E7 protein share the capacity to disrupt the interaction between transcription factor E2F and the retinoblastoma gene product. *Proc Natl Acad Sci U S A* **89:** 4549-4553

Chen G, Stenlund A (2002) Sequential and ordered assembly of E1 initiator complexes on the papillomavirus origin of DNA replication generates progressive structural changes related to melting. *Mol Cell Biol* **22:** 7712-7720

Cheng S, Schmidt-Grimminger DC, Murant T, Broker TR, Chow LT (1995) Differentiation-dependent up-regulation of the human papillomavirus E7 gene reactivates cellular DNA replication in suprabasal differentiated keratinocytes. *Genes Dev* **9:** 2335-2349

Chien WM, Parker JN, Schmidt-Grimminger DC, Broker TR, Chow LT (2000) Casein kinase II phosphorylation of the human papillomavirus-18 E7 protein is critical for promoting S-phase entry. *Cell Growth Differ* **11:** 425-435

Chung HK, Cheong C, Song J, Lee HW (2005) Extratelomeric functions of telomerase. *Current molecular medicine* **5:** 233-241

Cobrinik D (2005) Pocket proteins and cell cycle control. *Oncogene* **24:** 2796-2809

Conger KL, Liu JS, Kuo SR, Chow LT, Wang TS (1999) Human papillomavirus DNA replication. Interactions between the viral E1 protein and two subunits of human dna polymerase alpha/primase. *J Biol Chem* **274:** 2696-2705

Cooper EM, Hudson AW, Amos J, Wagstaff J, Howley PM (2004) Biochemical analysis of Angelman syndrome-associated mutations in the E3 ubiquitin ligase E6-associated protein. *J Biol Chem* **279:** 41208-41217

Corden SA, Sant-Cassia LJ, Easton AJ, Morris AG (1999) The integration of HPV-18 DNA in cervical carcinoma. *Mol Pathol* **52:** 275-282

Cripe TP, Haugen TH, Turk JP, Tabatabai F, Schmid PG, 3rd, Durst M, Gissmann L, Roman A, Turek LP (1987) Transcriptional regulation of the human papillomavirus-16 E6-E7 promoter by a keratinocyte-dependent enhancer, and by viral E2 trans-activator and repressor gene products: implications for cervical carcinogenesis. *Embo J* **6:** 3745-3753

Dao LD, Duffy A, Van Tine BA, Wu SY, Chiang CM, Broker TR, Chow LT (2006) Dynamic localization of the human papillomavirus type 11 origin binding protein E2 through mitosis while in association with the spindle apparatus. *J Virol* **80:** 4792-4800

Davies R, Hicks R, Crook T, Morris J, Vousden K (1993) Human papillomavirus type 16 E7 associates with a histone H1 kinase and with p107 through sequences necessary for transformation. *J Virol* **67:** 2521-2528

Davy CE, Jackson DJ, Raj K, Peh WL, Southern SA, Das P, Sorathia R, Laskey P, Middleton K, Nakahara T, Wang Q, Masterson PJ, Lambert PF, Cuthill S, Millar JB, Doorbar J (2005) Human papillomavirus type 16 E1 E4-induced G2 arrest is associated with cytoplasmic retention of active Cdk1/cyclin B1 complexes. *J Virol* **79:** 3998-4011

Day PM, Baker CC, Lowy DR, Schiller JT (2004) Establishment of papillomavirus infection is enhanced by promyelocytic leukemia protein (PML) expression. *Proc Natl Acad Sci U S A* **101:** 14252-14257

Day PM, Lowy DR, Schiller JT (2003) Papillomaviruses infect cells via a clathrin-dependent pathway. *Virology* **307:** 1-11

Day PM, Roden RB, Lowy DR, Schiller JT (1998) The papillomavirus minor capsid protein, L2, induces localization of the major capsid protein, L1, and the viral transcription/replication protein, E2, to PML oncogenic domains. *J Virol* **72:** 142-150

De Geest K, Turyk ME, Hosken MI, Hudson JB, Laimins LA, Wilbanks GD (1993) Growth and differentiation of human papillomavirus type 31b positive human cervical cell lines. *Gynecologic oncology* **49:** 303-310

Demeret C, Garcia-Carranca A, Thierry F (2003) Transcription-independent triggering of the extrinsic pathway of apoptosis by human papillomavirus 18 E2 protein. *Oncogene* **22:** 168-175

Demers GW, Espling E, Harry JB, Etscheid BG, Galloway DA (1996) Abrogation of growth arrest signals by human papillomavirus type 16 E7 is mediated by sequences required for transformation. *J Virol* **70:** 6862-6869

Demers GW, Halbert CL, Galloway DA (1994) Elevated wild-type p53 protein levels in human epithelial cell lines immortalized by the human papillomavirus type 16 E7 gene. *Virology* **198:** 169-174

Deng W, Lin BY, Jin G, Wheeler CG, Ma T, Harper JW, Broker TR, Chow LT (2004) Cyclin/CDK regulates the nucleocytoplasmic localization of the human papillomavirus E1 DNA helicase. *J Virol* **78:** 13954-13965

Dey A, Chitsaz F, Abbasi A, Misteli T, Ozato K (2003) The double bromodomain protein Brd4 binds to acetylated chromatin during interphase and mitosis. *Proc Natl Acad Sci U S A* **100:** 8758-8763

Donaldson MM, Boner W, Morgan IM (2007) TopBP1 regulates human papillomavirus type 16 E2 interaction with chromatin. *J Virol* **81:** 4338-4342

Donehower LA, Harvey M, Slagle BL, McArthur MJ, Montgomery CA, Jr., Butel JS, Bradley A (1992) Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature* **356:** 215-221

Dong G, Broker TR, Chow LT (1994) Human papillomavirus type 11 E2 proteins repress the homologous E6 promoter by interfering with the binding of host transcription factors to adjacent elements. *J Virol* **68:** 1115-1127

Doorbar J (2005) The papillomavirus life cycle. *J Clin Virol* **32 Suppl 1:** S7-15

Doorbar J (2006) Molecular biology of human papillomavirus infection and cervical cancer. *Clin Sci (Lond)* **110:** 525-541

Doorbar J, Elston RC, Napthine S, Raj K, Medcalf E, Jackson D, Coleman N, Griffin HM, Masterson P, Stacey S, Mengistu Y, Dunlop J (2000) The E1E4 protein of human papillomavirus type 16 associates with a putative RNA helicase through sequences in its C terminus. *J Virol* **74:** 10081-10095

Doorbar J, Ely S, Sterling J, McLean C, Crawford L (1991) Specific interaction between HPV-16 E1-E4 and cytokeratins results in collapse of the epithelial cell intermediate filament network. *Nature* **352:** 824-827

Doorbar J, Parton A, Hartley K, Banks L, Crook T, Stanley M, Crawford L (1990) Detection of novel splicing patterns in a HPV16-containing keratinocyte cell line. *Virology* **178:** 254-262

Dowhanick JJ, McBride AA, Howley PM (1995) Suppression of cellular proliferation by the papillomavirus E2 protein. *J Virol* **69:** 7791-7799

Duensing A, Liu Y, Spardy N, Bartoli K, Tseng M, Kwon JA, Teng X, Duensing S (2007) RNA polymerase II transcription is required for human papillomavirus type 16 E7- and hydroxyurea-induced centriole overduplication. *Oncogene* **26:** 215-223

Duensing S, Duensing A, Crum CP, Munger K (2001a) Human papillomavirus type 16 E7 oncoprotein-induced abnormal centrosome synthesis is an early event in the evolving malignant phenotype. *Cancer Res* **61:** 2356-2360

Duensing S, Duensing A, Flores ER, Do A, Lambert PF, Munger K (2001b) Centrosome abnormalities and genomic instability by episomal expression of human papillomavirus type 16 in raft cultures of human keratinocytes. *J Virol* **75:** 7712-7716

Duensing S, Duensing A, Lee DC, Edwards KM, Piboonniyom SO, Manuel E, Skaltsounis L, Meijer L, Munger K (2004) Cyclin-dependent kinase inhibitor indirubin-3'-oxime selectively inhibits human papillomavirus type 16 E7-induced numerical centrosome anomalies. *Oncogene* **23:** 8206-8215

Duensing S, Lee LY, Duensing A, Basile J, Piboonniyom S, Gonzalez S, Crum CP, Munger K (2000) The human papillomavirus type 16 E6 and E7 oncoproteins cooperate to induce mitotic defects and genomic instability by uncoupling centrosome duplication from the cell division cycle. *Proc Natl Acad Sci U S A* **97:** 10002-10007

Duensing S, Munger K (2002a) The human papillomavirus type 16 E6 and E7 oncoproteins independently induce numerical and structural chromosome instability. *Cancer Res* **62:** 7075-7082

Duensing S, Munger K (2002b) Human papillomaviruses and centrosome duplication errors: modeling the origins of genomic instability. *Oncogene* **21:** 6241-6248

Duensing S, Munger K (2003) Human papillomavirus type 16 E7 oncoprotein can induce abnormal centrosome duplication through a mechanism independent of inactivation of retinoblastoma protein family members. *J Virol* **77:** 12331-12335

Dyson N, Howley PM, Munger K, Harlow E (1989) The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. *Science* **243:** 934-937

Edmonds C, Vousden KH (1989) A point mutational analysis of human papillomavirus type 16 E7 protein. *J Virol* **63:** 2650-2656

Everett RD (2001) DNA viruses and viral proteins that interact with PML nuclear bodies. *Oncogene* **20:** 7266-7273

Favre-Bonvin A, Reynaud C, Kretz-Remy C, Jalinot P (2005) Human papillomavirus type 18 E6 protein binds the cellular PDZ protein TIP-2/GIPC, which is involved in transforming growth factor beta signaling and triggers its degradation by the proteasome. *J Virol* **79:** 4229-4237

Felsani A, Mileo AM, Paggi MG (2006) Retinoblastoma family proteins as key targets of the small DNA virus oncoproteins. *Oncogene* **25:** 5277-5285

Fergusson D, Campo MS (1998) PEF-1, an epithelial cell transcription factor which activates the long control region of human papillomavirus type 16, is glycosylated with N-acetylglucosamine. *J Gen Virol* **79 (Pt 11):** 2753-2760

Filippova M, Parkhurst L, Duerksen-Hughes PJ (2004) The human papillomavirus 16 E6 protein binds to Fas-associated death domain and protects cells from Fas-triggered apoptosis. *J Biol Chem* **279:** 25729-25744

Filippova M, Song H, Connolly JL, Dermody TS, Duerksen-Hughes PJ (2002) The human papillomavirus 16 E6 protein binds to tumor necrosis factor (TNF) R1 and protects cells from TNF-induced apoptosis. *J Biol Chem* **277:** 21730-21739

Firzlaff JM, Galloway DA, Eisenman RN, Luscher B (1989) The E7 protein of human papillomavirus type 16 is phosphorylated by casein kinase II. *New Biol* **1:** 44-53

Firzlaff JM, Luscher B, Eisenman RN (1991) Negative charge at the casein kinase II phosphorylation site is important for transformation but not for Rb protein binding by the E7 protein of human papillomavirus type 16. *Proc Natl Acad Sci U S A* **88:** 5187-5191

Flores ER, Allen-Hoffmann BL, Lee D, Lambert PF (2000) The human papillomavirus type 16 E7 oncogene is required for the productive stage of the viral life cycle. *J Virol* **74:** 6622-6631

Flores ER, Lambert PF (1997) Evidence for a switch in the mode of human papillomavirus type 16 DNA replication during the viral life cycle. *J Virol* **71:** 7167-7179

Francis DA, Schmid SI, Howley PM (2000) Repression of the integrated papillomavirus E6/E7 promoter is required for growth suppression of cervical cancer cells. *J Virol* **74:** 2679-2686

Freed E, Lacey KR, Huie P, Lyapina SA, Deshaies RJ, Stearns T, Jackson PK (1999) Components of an SCF ubiquitin ligase localize to the centrosome and regulate the centrosome duplication cycle. *Genes Dev* **13:** 2242-2257

Fukasawa K (2002) Introduction. Centrosome. *Oncogene* **21:** 6140-6145

Funk JO, Waga S, Harry JB, Espling E, Stillman B, Galloway DA (1997) Inhibition of CDK activity and PCNA-dependent DNA replication by p21 is blocked by interaction with the HPV-16 E7 oncoprotein. *Genes Dev* **11:** 2090-2100

Gardiol D, Kuhne C, Glaunsinger B, Lee SS, Javier R, Banks L (1999) Oncogenic human papillomavirus E6 proteins target the discs large tumour suppressor for proteasome-mediated degradation. *Oncogene* **18:** 5487-5496

Genther SM, Sterling S, Duensing S, Munger K, Sattler C, Lambert PF (2003) Quantitative role of the human papillomavirus type 16 E5 gene during the productive stage of the viral life cycle. *J Virol* **77:** 2832-2842

Gillison ML, Shah KV (2001) Human papillomavirus-associated head and neck squamous cell carcinoma: mounting evidence for an etiologic role for human papillomavirus in a subset of head and neck cancers. *Current opinion in oncology* **13:** 183-188

Glaunsinger BA, Lee SS, Thomas M, Banks L, Javier R (2000) Interactions of the PDZ-protein MAGI-1 with adenovirus E4-ORF1 and high-risk papillomavirus E6 oncoproteins. *Oncogene* **19:** 5270-5280

Gloss B, Bernard HU (1990) The E6/E7 promoter of human papillomavirus type 16 is activated in the absence of E2 proteins by a sequence-aberrant Sp1 distal element. *J Virol* **64:** 5577-5584

Goodwin EC, DiMaio D (2000) Repression of human papillomavirus oncogenes in HeLa cervical carcinoma cells causes the orderly reactivation of dormant tumor suppressor pathways. *Proc Natl Acad Sci U S A* **97:** 12513-12518

Gopalakrishnan V, Sheahan L, Khan SA (1999) DNA replication specificity and functional E2 interaction of the E1 proteins of human papillomavirus types 1a and 18 are determined by their carboxyl-terminal halves. *Virology* **256:** 330-339

Grm HS, Banks L (2004) Degradation of hDlg and MAGIs by human papillomavirus E6 is E6-AP-independent. *J Gen Virol* **85:** 2815-2819

Grm HS, Massimi P, Gammoh N, Banks L (2005) Crosstalk between the human papillomavirus E2 transcriptional activator and the E6 oncoprotein. *Oncogene* **24:** 5149-5164

Gross-Mesilaty S, Reinstein E, Bercovich B, Tobias KE, Schwartz AL, Kahana C, Ciechanover A (1998) Basal and human papillomavirus E6 oncoprotein-induced degradation of Myc proteins by the ubiquitin pathway. *Proc Natl Acad Sci U S A* **95:** 8058-8063

Guccione E, Massimi P, Bernat A, Banks L (2002) Comparative analysis of the intracellular location of the high- and low-risk human papillomavirus oncoproteins. *Virology* **293:** 20-25

Hadaschik D, Hinterkeuser K, Oldak M, Pfister HJ, Smola-Hess S (2003) The Papillomavirus E2 protein binds to and synergizes with C/EBP factors involved in keratinocyte differentiation. *J Virol* **77:** 5253-5265

Hanahan D, Weinberg RA (2000) The hallmarks of cancer. *Cell* **100:** 57-70

Handa K, Yugawa T, Narisawa-Saito M, Ohno S, Fujita M, Kiyono T (2007) E6AP-dependent degradation of DLG4/PSD95 by high-risk human papillomavirus type 18 E6 protein. *J Virol* **81:** 1379-1389

Harwood CA, Proby CM (2002) Human papillomaviruses and non-melanoma skin cancer. *Current opinion in infectious diseases* **15:** 101-114

He W, Staples D, Smith C, Fisher C (2003) Direct activation of cyclin-dependent kinase 2 by human papillomavirus E7. *J Virol* **77:** 10566-10574

Heck DV, Yee CL, Howley PM, Munger K (1992) Efficiency of binding the retinoblastoma protein correlates with the transforming capacity of the E7 oncoproteins of the human papillomaviruses. *Proc Natl Acad Sci U S A* **89:** 4442-4446

Heino P, Zhou J, Lambert PF (2000) Interaction of the papillomavirus transcription/replication factor, E2, and the viral capsid protein, L2. *Virology* **276:** 304-314

Helt AM, Galloway DA (2001) Destabilization of the retinoblastoma tumor suppressor by human papillomavirus type 16 E7 is not sufficient to overcome cell cycle arrest in human keratinocytes. *J Virol* **75:** 6737-6747

Hemminki K, Dong C, Vaittinen P (1999) Familial risks in cervical cancer: is there a hereditary component? *Int J Cancer* **82:** 775-781

Herber R, Liem A, Pitot H, Lambert PF (1996) Squamous epithelial hyperplasia and carcinoma in mice transgenic for the human papillomavirus type 16 E7 oncogene. *J Virol* **70:** 1873-1881

Hibma MH, Raj K, Ely SJ, Stanley M, Crawford L (1995) The interaction between human papillomavirus type 16 E1 and E2 proteins is blocked by an antibody to the N-terminal region of E2. *Eur J Biochem* **229:** 517-525

Hinchcliffe EH, Sluder G (2001) "It takes two to tango": understanding how centrosome duplication is regulated throughout the cell cycle. *Genes Dev* **15:** 1167-1181

Hines CS, Meghoo C, Shetty S, Biburger M, Brenowitz M, Hegde RS (1998) DNA structure and flexibility in the sequence-specific binding of papillomavirus E2 proteins. *J Mol Biol* **276:** 809-818

Horner SM, DeFilippis RA, Manuelidis L, DiMaio D (2004) Repression of the human papillomavirus E6 gene initiates p53-dependent, telomerase-independent senescence and apoptosis in HeLa cervical carcinoma cells. *J Virol* **78:** 4063-4073

Huang SM, McCance DJ (2002) Down regulation of the interleukin-8 promoter by human papillomavirus type 16 E6 and E7 through effects on CREB binding protein/p300 and P/CAF. *J Virol* **76:** 8710-8721

Huh KW, DeMasi J, Ogawa H, Nakatani Y, Howley PM, Munger K (2005) Association of the human papillomavirus type 16 E7 oncoprotein with the 600-kDa retinoblastoma protein-associated factor, p600. *Proc Natl Acad Sci U S A* **102:** 11492-11497

Hwang SG, Lee D, Kim J, Seo T, Choe J (2002) Human papillomavirus type 16 E7 binds to E2F1 and activates E2F1-driven transcription in a retinoblastoma protein-independent manner. *J Biol Chem* **277:** 2923-2930

Imai Y, Matsushima Y, Sugimura T, Terada M (1991) Purification and characterization of human papillomavirus type 16 E7 protein with preferential binding capacity to the underphosphorylated form of retinoblastoma gene product. *J Virol* **65:** 4966-4972

Incassati A, Patel D, McCance DJ (2006) Induction of tetraploidy through loss of p53 and upregulation of Plk1 by human papillomavirus type-16 E6. *Oncogene* **25:** 2444-2451

Iyer NG, Ozdag H, Caldas C (2004) p300/CBP and cancer. *Oncogene* **23:** 4225-4231

James MA, Lee JH, Klingelhutz AJ (2006) Human papillomavirus type 16 E6 activates NF-kappaB, induces cIAP-2 expression, and protects against apoptosis in a PDZ binding motif-dependent manner. *J Virol* **80:** 5301-5307

Jenson AB, Kurman RJ, Lancaster WD (1991) Tissue effects of and host response to human papillomavirus infection. *Dermatologic clinics* **9:** 203-209

Jewers RJ, Hildebrandt P, Ludlow JW, Kell B, McCance DJ (1992) Regions of human papillomavirus type 16 E7 oncoprotein required for immortalization of human keratinocytes. *J Virol* **66:** 1329-1335

Jing M, Bohl J, Brimer N, Kinter M, Vande Pol SB (2007) Degradation of tyrosine phosphatase PTPN3 (PTPH1) by association with oncogenic human papillomavirus E6 proteins. *J Virol* **81:** 2231-2239

Jones DL, Alani RM, Munger K (1997) The human papillomavirus E7 oncoprotein can uncouple cellular differentiation and proliferation in human keratinocytes by abrogating p21Cip1-mediated inhibition of cdk2. *Genes Dev* **11:** 2101-2111

Jones DL, Munger K (1997) Analysis of the p53-mediated G1 growth arrest pathway in cells expressing the human papillomavirus type 16 E7 oncoprotein. *J Virol* **71:** 2905-2912

Jones S, Thornton JM (1996) Principles of protein-protein interactions. *Proc Natl Acad Sci U S A* **93:** 13-20

Kaznelson DW, Bruun S, Monrad A, Gjerlov S, Birk J, Ropke C, Norrild B (2004) Simultaneous human papilloma virus type 16 E7 and cdk inhibitor p21 expression induces apoptosis and cathepsin B activation. *Virology* **320:** 301-312

Kee SH, Choi YO, Song YS, Lee HP, Chang WH (1998) Identification of antigenic differences between the phosphorylated and nonphosphorylated forms of the E7 protein of human papillomavirus type 16. *Journal of medical virology* **54:** 129-134

Kelley ML, Keiger KE, Lee CJ, Huibregtse JM (2005) The global transcriptional effects of the human papillomavirus E6 protein in cervical carcinoma cell lines are mediated by the E6AP ubiquitin ligase. *J Virol* **79:** 3737-3747

Khosravi R, Maya R, Gottlieb T, Oren M, Shiloh Y, Shkedy D (1999) Rapid ATM-dependent phosphorylation of MDM2 precedes p53 accumulation in response to DNA damage. *Proc Natl Acad Sci U S A* **96:** 14973-14977

Kim K, Garner-Hamrick PA, Fisher C, Lee D, Lambert PF (2003) Methylation patterns of papillomavirus DNA, its influence on E2 function, and implications in viral infection. *J Virol* **77:** 12450-12459

Kirnbauer R, Taub J, Greenstone H, Roden R, Durst M, Gissmann L, Lowy DR, Schiller JT (1993) Efficient self-assembly of human papillomavirus type 16 L1 and L1-L2 into virus-like particles. *J Virol* **67:** 6929-6936

Klein G (2002) Perspectives in studies of human tumor viruses. *Front Biosci* **7:** d268-274

Klingelhutz AJ, Foster SA, McDougall JK (1996) Telomerase activation by the E6 gene product of human papillomavirus type 16. *Nature* **380:** 79-82

Klucevsek K, Wertz M, Lucchi J, Leszczynski A, Moroianu J (2007) Characterization of the nuclear localization signal of high risk HPV16 E2 protein. *Virology* **360:** 191-198

Krithivas A, Fujimuro M, Weidner M, Young DB, Hayward SD (2002) Protein interactions targeting the latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus to cell chromosomes. *J Virol* **76:** 11596-11604

Kuballa P, Matentzoglu K, Scheffner M (2007) The role of the ubiquitin ligase E6-AP in human papillomavirus E6-mediated degradation of PDZ domain-containing proteins. *J Biol Chem* **282:** 65-71

Kurg R, Sild K, Ilves A, Sepp M, Ustav M (2005) Association of bovine papillomavirus E2 protein with nuclear structures in vivo. *J Virol* **79:** 10528-10539

Kurki S, Latonen L, Laiho M (2003) Cellular stress and DNA damage invoke temporally distinct Mdm2, p53 and PML complexes and damage-specific nuclear relocalization. *Journal of cell science* **116:** 3917-3925

Lai MC, Teh BH, Tarn WY (1999) A human papillomavirus E2 transcriptional activator. The interactions with cellular splicing factors and potential function in pre-mRNA processing. *J Biol Chem* **274:** 11832-11841

Lee C, Laimins LA (2004) Role of the PDZ domain-binding motif of the oncoprotein E6 in the pathogenesis of human papillomavirus type 31. *J Virol* **78:** 12366-12377

Lee SS, Glaunsinger B, Mantovani F, Banks L, Javier RT (2000) Multi-PDZ domain protein MUPP1 is a cellular target for both adenovirus E4-ORF1 and high-risk papillomavirus type 18 E6 oncoproteins. *J Virol* **74:** 9680-9693

Lengauer C, Kinzler KW, Vogelstein B (1998) Genetic instabilities in human cancers. *Nature* **396:** 643-649

Lentini L, Iovino F, Amato A, Di Leonardo A (2006) Centrosome amplification induced by hydroxyurea leads to aneuploidy in pRB deficient human and mouse fibroblasts. *Cancer letters* **238:** 153-160

Lepik D, Ilves I, Kristjuhan A, Maimets T, Ustav M (1998) p53 protein is a suppressor of papillomavirus DNA amplificational replication. *J Virol* **72:** 6822-6831

Li B, Dou QP (2000) Bax degradation by the ubiquitin/proteasome-dependent pathway: involvement in tumor survival and progression. *Proc Natl Acad Sci U S A* **97:** 3850-3855

Lin BY, Ma T, Liu JS, Kuo SR, Jin G, Broker TR, Harper JW, Chow LT (2000) HeLa cells are phenotypically limiting in cyclin E/CDK2 for efficient human papillomavirus DNA replication. *J Biol Chem* **275:** 6167-6174

Lin BY, Makhov AM, Griffith JD, Broker TR, Chow LT (2002) Chaperone proteins abrogate inhibition of the human papillomavirus (HPV) E1 replicative helicase by the HPV E2 protein. *Mol Cell Biol* **22:** 6592-6604

Liu X, Clements A, Zhao K, Marmorstein R (2006) Structure of the human Papillomavirus E7 oncoprotein and its mechanism for inactivation of the retinoblastoma tumor suppressor. *J Biol Chem* **281:** 578-586

Liu X, Yuan H, Fu B, Disbrow GL, Apolinario T, Tomaic V, Kelley ML, Baker CC, Huibregtse J, Schlegel R (2005) The E6AP ubiquitin ligase is required for transactivation of the hTERT promoter by the human papillomavirus E6 oncoprotein. *J Biol Chem* **280:** 10807-10816

Liu Y, Chen JJ, Gao Q, Dalal S, Hong Y, Mansur CP, Band V, Androphy EJ (1999) Multiple functions of human papillomavirus type 16 E6 contribute to the immortalization of mammary epithelial cells. *J Virol* **73:** 7297-7307

Luscher-Firzlaff JM, Westendorf JM, Zwicker J, Burkhardt H, Henriksson M, Muller R, Pirollet F, Luscher B (1999) Interaction of the fork head domain transcription factor MPP2 with the human papilloma virus 16 E7 protein: enhancement of transformation and transactivation. *Oncogene* **18:** 5620-5630

Ma T, Zou N, Lin BY, Chow LT, Harper JW (1999) Interaction between cyclin-dependent kinases and human papillomavirus replication-initiation protein E1 is required for efficient viral replication. *Proc Natl Acad Sci U S A* **96:** 382-387

Mack DH, Laimins LA (1991) A keratinocyte-specific transcription factor, KRF-1, interacts with AP-1 to activate expression of human papillomavirus type 18 in squamous epithelial cells. *Proc Natl Acad Sci U S A* **88:** 9102-9106

Magnusson PK, Sparen P, Gyllensten UB (1999) Genetic link to cervical tumours. *Nature* **400:** 29-30

Mannhardt B, Weinzimer SA, Wagner M, Fiedler M, Cohen P, Jansen-Durr P, Zwerschke W (2000) Human papillomavirus type 16 E7 oncoprotein binds and inactivates growth-inhibitory insulin-like growth factor binding protein 3. *Mol Cell Biol* **20:** 6483-6495

Mantovani F, Banks L (2001) The human papillomavirus E6 protein and its contribution to malignant progression. *Oncogene* **20:** 7874-7887

Mantovani F, Gostissa M, Collavin L, Del Sal G (2004) KeePin' the p53 family in good shape. *Cell Cycle* **3:** 905-911

Massague J (2004) G1 cell-cycle control and cancer. *Nature* **432:** 298-306

Massimi P, Banks L (2000) Differential phosphorylation of the HPV-16 E7 oncoprotein during the cell cycle. *Virology* **276:** 388-394

Massimi P, Gammoh N, Thomas M, Banks L (2004) HPV E6 specifically targets different cellular pools of its PDZ domain-containing tumour suppressor substrates for proteasome-mediated degradation. *Oncogene* **23:** 8033-8039

Massimi P, Pim D, Banks L (1997) Human papillomavirus type 16 E7 binds to the conserved carboxy-terminal region of the TATA box binding protein and this contributes to E7 transforming activity. *J Gen Virol* **78 (Pt 10):** 2607-2613

Massimi P, Pim D, Bertoli C, Bouvard V, Banks L (1999) Interaction between the HPV-16 E2 transcriptional activator and p53. *Oncogene* **18:** 7748-7754

Massimi P, Pim D, Storey A, Banks L (1996) HPV-16 E7 and adenovirus E1a complex formation with TATA box binding protein is enhanced by casein kinase II phosphorylation. *Oncogene* **12:** 2325-2330

Masterson PJ, Stanley MA, Lewis AP, Romanos MA (1998) A C-terminal helicase domain of the human papillomavirus E1 protein binds E2 and the DNA polymerase alpha-primase p68 subunit. *J Virol* **72:** 7407-7419

Matlashewski G, Schneider J, Banks L, Jones N, Murray A, Crawford L (1987) Human papillomavirus type 16 DNA cooperates with activated ras in transforming primary cells. *Embo J* **6:** 1741-1746

Matsumoto Y, Nakagawa S, Yano T, Takizawa S, Nagasaka K, Nakagawa K, Minaguchi T, Wada O, Ooishi H, Matsumoto K, Yasugi T, Kanda T, Huibregtse JM, Taketani Y (2006) Involvement of a cellular ubiquitin-protein ligase E6AP in the ubiquitin-mediated degradation of extensive substrates of high-risk human papillomavirus E6. *Journal of medical virology* **78:** 501-507

Mayo LD, Donner DB (2001) A phosphatidylinositol 3-kinase/Akt pathway promotes translocation of Mdm2 from the cytoplasm to the nucleus. *Proc Natl Acad Sci U S A* **98:** 11598-11603

Mayo LD, Turchi JJ, Berberich SJ (1997) Mdm-2 phosphorylation by DNA-dependent protein kinase prevents interaction with p53. *Cancer Res* **57:** 5013-5016

Mazzarelli JM, Atkins GB, Geisberg JV, Ricciardi RP (1995) The viral oncoproteins Ad5 E1A, HPV16 E7 and SV40 TAg bind a common region of the TBP-associated factor-110. *Oncogene* **11:** 1859-1864

McBride AA, McPhillips MG, Oliveira JG (2004) Brd4: tethering, segregation and beyond. *Trends Microbiol* **12:** 527-529

McBride AA, Oliveira JG, McPhillips MG (2006) Partitioning viral genomes in mitosis: same idea, different targets. *Cell Cycle* **5:** 1499-1502

McCance DJ (2005) Human papillomaviruses and cell signaling. *Sci STKE* **2005:** pe29

McIntyre MC, Frattini MG, Grossman SR, Laimins LA (1993) Human papillomavirus type 18 E7 protein requires intact Cys-X-X-Cys motifs for zinc binding, dimerization, and transformation but not for Rb binding. *J Virol* **67:** 3142-3150

McIntyre MC, Ruesch MN, Laimins LA (1996) Human papillomavirus E7 oncoproteins bind a single form of cyclin E in a complex with cdk2 and p107. *Virology* **215:** 73-82

McMurray HR, McCance DJ (2004) Degradation of p53, not telomerase activation, by E6 is required for bypass of crisis and immortalization by human papillomavirus type 16 E6/E7. *J Virol* **78:** 5698-5706

McPhillips MG, Oliveira JG, Spindler JE, Mitra R, McBride AA (2006) Brd4 is required for e2-mediated transcriptional activation but not genome partitioning of all papillomaviruses. *J Virol* **80:** 9530-9543

McPhillips MG, Ozato K, McBride AA (2005) Interaction of bovine papillomavirus E2 protein with Brd4 stabilizes its association with chromatin. *J Virol* **79:** 8920-8932

Meek DW, Knippschild U (2003) Posttranslational modification of MDM2. *Mol Cancer Res* **1:** 1017-1026

Menges CW, Baglia LA, Lapoint R, McCance DJ (2006) Human papillomavirus type 16 E7 up-regulates AKT activity through the retinoblastoma protein. *Cancer Res* **66:** 5555-5559

Meraldi P, Lukas J, Fry AM, Bartek J, Nigg EA (1999) Centrosome duplication in mammalian somatic cells requires E2F and Cdk2-cyclin A. *Nature cell biology* **1:** 88-93

Meraldi P, Nigg EA (2002) The centrosome cycle. *FEBS letters* **521:** 9-13

Middleton K, Peh W, Southern S, Griffin H, Sotlar K, Nakahara T, El-Sherif A, Morris L, Seth R, Hibma M, Jenkins D, Lambert P, Coleman N, Doorbar J (2003) Organization of human papillomavirus productive cycle during neoplastic progression provides a basis for selection of diagnostic markers. *J Virol* **77:** 10186-10201

Mohr IJ, Clark R, Sun S, Androphy EJ, MacPherson P, Botchan MR (1990) Targeting the E1 replication protein to the papillomavirus origin of replication by complex formation with the E2 transactivator. *Science* **250:** 1694-1699

Munger K, Baldwin A, Edwards KM, Hayakawa H, Nguyen CL, Owens M, Grace M, Huh K (2004) Mechanisms of human papillomavirus-induced oncogenesis. *J Virol* **78:** 11451-11460

Muratani M, Tansey WP (2003) How the ubiquitin-proteasome system controls transcription. *Nat Rev Mol Cell Biol* **4:** 192-201

Nakagawa S, Huibregtse JM (2000) Human scribble (Vartul) is targeted for ubiquitin-mediated degradation by the high-risk papillomavirus E6 proteins and the E6AP ubiquitin-protein ligase. *Mol Cell Biol* **20:** 8244-8253

Nakagawa S, Watanabe S, Yoshikawa H, Taketani Y, Yoshiike K, Kanda T (1995) Mutational analysis of human papillomavirus type 16 E6 protein: transforming function for human cells and degradation of p53 in vitro. *Virology* **212:** 535-542

Nakahara T, Nishimura A, Tanaka M, Ueno T, Ishimoto A, Sakai H (2002) Modulation of the cell division cycle by human papillomavirus type 18 E4. *J Virol* **76:** 10914-10920

Nakahara T, Peh WL, Doorbar J, Lee D, Lambert PF (2005) Human papillomavirus type 16 E1circumflexE4 contributes to multiple facets of the papillomavirus life cycle. *J Virol* **79:** 13150-13165

Nakayama K, Nagahama H, Minamishima YA, Matsumoto M, Nakamichi I, Kitagawa K, Shirane M, Tsunematsu R, Tsukiyama T, Ishida N, Kitagawa M, Nakayama K, Hatakeyama S (2000) Targeted disruption of Skp2 results in accumulation of cyclin E and p27(Kip1), polyploidy and centrosome overduplication. *Embo J* **19:** 2069-2081

Nakayama KI, Nakayama K (2005) Regulation of the cell cycle by SCF-type ubiquitin ligases. *Seminars in cell & developmental biology* **16:** 323-333

Nasseri M, Hirochika R, Broker TR, Chow LT (1987) A human papilloma virus type 11 transcript encoding an E1--E4 protein. *Virology* **159:** 433-439

Nguyen DX, Baglia LA, Huang SM, Baker CM, McCance DJ (2004) Acetylation regulates the differentiation-specific functions of the retinoblastoma protein. *Embo J* **23:** 1609-1618

Nguyen DX, McCance DJ (2005) Role of the retinoblastoma tumor suppressor protein in cellular differentiation. *Journal of cellular biochemistry* **94:** 870-879

Nguyen MM, Nguyen ML, Caruana G, Bernstein A, Lambert PF, Griep AE (2003) Requirement of PDZ-containing proteins for cell cycle regulation and differentiation in the mouse lens epithelium. *Mol Cell Biol* **23:** 8970-8981

Oh KJ, Kalinina A, Wang J, Nakayama K, Nakayama KI, Bagchi S (2004a) The papillomavirus E7 oncoprotein is ubiquitinated by UbcH7 and Cullin 1- and Skp2-containing E3 ligase. *J Virol* **78:** 5338-5346

Oh ST, Longworth MS, Laimins LA (2004b) Roles of the E6 and E7 proteins in the life cycle of low-risk human papillomavirus type 11. *J Virol* **78:** 2620-2626

Ohlenschlager O, Seiboth T, Zengerling H, Briese L, Marchanka A, Ramachandran R, Baum M, Korbas M, Meyer-Klaucke W, Durst M, Gorlach M (2006) Solution structure of the partially folded high-risk human papilloma virus 45 oncoprotein E7. *Oncogene* **25:** 5953-5959

Oliveira JG, Colf LA, McBride AA (2006) Variations in the association of papillomavirus E2 proteins with mitotic chromosomes. *Proc Natl Acad Sci U S A* **103:** 1047-1052

Palefsky J (2006) Biology of HPV in HIV infection. *Advances in dental research* **19:** 99-105

Pan W, Datta A, Adami GR, Raychaudhuri P, Bagchi S (2003) P19ARF inhibits the functions of the HPV16 E7 oncoprotein. *Oncogene* **22:** 5496-5503

Pang R, Lee TK, Poon RT, Fan ST, Wong KB, Kwong YL, Tse E (2007) Pin1 interacts with a specific serine-proline motif of hepatitis B virus X-protein to enhance hepatocarcinogenesis. *Gastroenterology* **132:** 1088-1103

Parish JL, Bean AM, Park RB, Androphy EJf (2006) ChlR1 Is Required for Loading Papillomavirus E2 onto Mitotic Chromosomes and Viral Genome Maintenance. *Molecular cell* **24:** 867-876

Park RB, Androphy EJ (2002) Genetic analysis of high-risk e6 in episomal maintenance of human papillomavirus genomes in primary human keratinocytes. *J Virol* **76:** 11359-11364

Parkin DM (2006) The global health burden of infection-associated cancers in the year 2002. *Int J Cancer* **118:** 3030-3044

Patel D, Huang SM, Baglia LA, McCance DJ (1999) The E6 protein of human papillomavirus type 16 binds to and inhibits co-activation by CBP and p300. *Embo J* **18:** 5061-5072

Patel D, Incassati A, Wang N, McCance DJ (2004) Human papillomavirus type 16 E6 and E7 cause polyploidy in human keratinocytes and up-regulation of G2-M-phase proteins. *Cancer Res* **64:** 1299-1306

Patrick DR, Oliff A, Heimbrook DC (1994) Identification of a novel retinoblastoma gene product binding site on human papillomavirus type 16 E7 protein. *J Biol Chem* **269:** 6842-6850

Peh WL, Brandsma JL, Christensen ND, Cladel NM, Wu X, Doorbar J (2004) The viral E4 protein is required for the completion of the cottontail rabbit papillomavirus productive cycle in vivo. *J Virol* **78:** 2142-2151

Pei XF, Qin NG, Meck JM, Schlegel R (1994) Keratinocytes immortalized by human papillomavirus-18 exhibit alterations dependent upon host genetic background and complexity of viral genes transfected. *Pathobiology* **62:** 43-52

Peitsaro P, Johansson B, Syrjanen S (2002) Integrated human papillomavirus type 16 is frequently found in cervical cancer precursors as demonstrated by a novel quantitative real-time PCR technique. *Journal of clinical microbiology* **40:** 886-891

Penrose KJ, Garcia-Alai M, de Prat-Gay G, McBride AA (2004) Casein Kinase II phosphorylation-induced conformational switch triggers degradation of the papillomavirus E2 protein. *J Biol Chem* **279:** 22430-22439

Petroski MD, Deshaies RJ (2005) Function and regulation of cullin-RING ubiquitin ligases. *Nat Rev Mol Cell Biol* **6:** 9-20

Petry KU, Scheffel D, Bode U, Gabrysiak T, Kochel H, Kupsch E, Glaubitz M, Niesert S, Kuhnle H, Schedel I (1994) Cellular immunodeficiency enhances the progression of human papillomavirus-associated cervical lesions. *Int J Cancer* **57:** 836-840

Phelps WC, Yee CL, Munger K, Howley PM (1988) The human papillomavirus type 16 E7 gene encodes transactivation and transformation functions similar to those of adenovirus E1A. *Cell* **53:** 539-547

Phelps WC, Yee CL, Munger K, Howley PM (1989) Functional and sequence similarities between HPV16 E7 and adenovirus E1A. *Current topics in microbiology and immunology* **144:** 153-166

Piccini A, Storey A, Massimi P, Banks L (1995) Mutations in the human papillomavirus type 16 E2 protein identify multiple regions of the protein involved in binding to E1. *J Gen Virol* **76 (Pt 11):** 2909-2913

Piirsoo M, Ustav E, Mandel T, Stenlund A, Ustav M (1996) Cis and trans requirements for stable episomal maintenance of the BPV-1 replicator. *Embo J* **15:** 1-11

Pim D, Collins M, Banks L (1992) Human papillomavirus type 16 E5 gene stimulates the transforming activity of the epidermal growth factor receptor. *Oncogene* **7:** 27-32

Pim D, Massimi P, Banks L (1997) Alternatively spliced HPV-18 E6* protein inhibits E6 mediated degradation of p53 and suppresses transformed cell growth. *Oncogene* **15:** 257-264

Pim D, Massimi P, Dilworth SM, Banks L (2005) Activation of the protein kinase B pathway by the HPV-16 E7 oncoprotein occurs through a mechanism involving interaction with PP2A. *Oncogene* **24:** 7830-7838

Pim D, Storey A, Thomas M, Massimi P, Banks L (1994) Mutational analysis of HPV-18 E6 identifies domains required for p53 degradation in vitro, abolition of p53 transactivation in vivo and immortalisation of primary BMK cells. *Oncogene* **9:** 1869-1876

Plug-DeMaggio AW, Sundsvold T, Wurscher MA, Koop JI, Klingelhutz AJ, McDougall JK (2004) Telomere erosion and chromosomal instability in cells expressing the HPV oncogene 16E6. *Oncogene* **23:** 3561-3571

Popescu NC, DiPaolo JA (1990) Integration of human papillomavirus 16 DNA and genomic rearrangements in immortalized human keratinocyte lines. *Cancer Res* **50:** 1316-1323

Prathapam T, Kuhne C, Banks L (2001) The HPV-16 E7 oncoprotein binds Skip and suppresses its transcriptional activity. *Oncogene* **20:** 7677-7685

Raj K, Berguerand S, Southern S, Doorbar J, Beard P (2004) E1 empty set E4 protein of human papillomavirus type 16 associates with mitochondria. *J Virol* **78:** 7199-7207

Rawls JA, Pusztai R, Green M (1990) Chemical synthesis of human papillomavirus type 16 E7 oncoprotein: autonomous protein domains for induction of cellular DNA synthesis and for trans activation. *J Virol* **64:** 6121-6129

Rehtanz M, Schmidt HM, Warthorst U, Steger G (2004) Direct interaction between nucleosome assembly protein 1 and the papillomavirus E2 proteins involved in activation of transcription. *Mol Cell Biol* **24:** 2153-2168

Reid G, Hubner MR, Metivier R, Brand H, Denger S, Manu D, Beaudouin J, Ellenberg J, Gannon F (2003) Cyclic, proteasome-mediated turnover of unliganded and liganded ERalpha on responsive promoters is an integral feature of estrogen signaling. *Molecular cell* **11:** 695-707

Reinstein E, Scheffner M, Oren M, Ciechanover A, Schwartz A (2000) Degradation of the E7 human papillomavirus oncoprotein by the ubiquitin-proteasome system: targeting via ubiquitination of the N-terminal residue. *Oncogene* **19:** 5944-5950

Riley RR, Duensing S, Brake T, Munger K, Lambert PF, Arbeit JM (2003) Dissection of human papillomavirus E6 and E7 function in transgenic mouse models of cervical carcinogenesis. *Cancer Res* **63:** 4862-4871

Roden R, Wu TC (2006) How will HPV vaccines affect cervical cancer? *Nature reviews* **6:** 753-763

Roden RB, Greenstone HL, Kirnbauer R, Booy FP, Jessie J, Lowy DR, Schiller JT (1996) In vitro generation and type-specific neutralization of a human papillomavirus type 16 virion pseudotype. *J Virol* **70:** 5875-5883

Rohlfs M, Winkenbach S, Meyer S, Rupp T, Durst M (1991) Viral transcription in human keratinocyte cell lines immortalized by human papillomavirus type-16. *Virology* **183:** 331-342

Romanczuk H, Howley PM (1992) Disruption of either the E1 or the E2 regulatory gene of human papillomavirus type 16 increases viral immortalization capacity. *Proc Natl Acad Sci U S A* **89:** 3159-3163

Saji S, Okumura N, Eguchi H, Nakashima S, Suzuki A, Toi M, Nozawa Y, Saji S, Hayashi S (2001) MDM2 enhances the function of estrogen receptor alpha in human breast cancer cells. *Biochemical and biophysical research communications* **281:** 259-265

Sanders CM, Stenlund A (1998) Recruitment and loading of the E1 initiator protein: an ATP-dependent process catalysed by a transcription factor. *Embo J* **17:** 7044-7055

Sang BC, Barbosa MS (1992) Increased E6/E7 transcription in HPV 18-immortalized human keratinocytes results from inactivation of E2 and additional cellular events. *Virology* **189:** 448-455

Sato H, Watanabe S, Furuno A, Yoshiike K (1989) Human papillomavirus type 16 E7 protein expressed in Escherichia coli and monkey COS-1 cells: immunofluorescence detection of the nuclear E7 protein. *Virology* **170:** 311-315

Schaeffer AJ, Nguyen M, Liem A, Lee D, Montagna C, Lambert PF, Ried T, Difilippantonio MJ (2004) E6 and E7 oncoproteins induce distinct patterns of chromosomal aneuploidy in skin tumors from transgenic mice. *Cancer Res* **64:** 538-546

Scheffner M, Huibregtse JM, Vierstra RD, Howley PM (1993) The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. *Cell* **75:** 495-505

Scheffner M, Munger K, Byrne JC, Howley PM (1991) The state of the p53 and retinoblastoma genes in human cervical carcinoma cell lines. *Proc Natl Acad Sci U S A* **88:** 5523-5527

Scheffner M, Werness BA, Huibregtse JM, Levine AJ, Howley PM (1990) The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell* **63:** 1129-1136

Schneider-Gadicke A, Schwarz E (1986) Different human cervical carcinoma cell lines show similar transcription patterns of human papillomavirus type 18 early genes. *Embo J* **5:** 2285-2292

Scholey JM, Brust-Mascher I, Mogilner A (2003) Cell division. *Nature* **422:** 746-752

Schuck S, Stenlund A (2005) Role of papillomavirus E1 initiator dimerization in DNA replication. *J Virol* **79:** 8661-8664

Seavey SE, Holubar M, Saucedo LJ, Perry ME (1999) The E7 oncoprotein of human papillomavirus type 16 stabilizes p53 through a mechanism independent of p19(ARF). *J Virol* **73:** 7590-7598

Sethi N, Palefsky J (2004) Transcriptional profiling of dysplastic lesions in K14-HPV16 transgenic mice using laser microdissection. *Faseb J* **18:** 1243-1245

Shinmura K, Bennett RA, Tarapore P, Fukasawa K (2007) Direct evidence for the role of centrosomally localized p53 in the regulation of centrosome duplication. *Oncogene* **26:** 2939-2944

Simonson SJ, Difilippantonio MJ, Lambert PF (2005) Two distinct activities contribute to human papillomavirus 16 E6's oncogenic potential. *Cancer Res* **65:** 8266-8273

Sin JI (2006) Human papillomavirus vaccines for the treatment of cervical cancer. *Expert review of vaccines* **5:** 783-792

Skiadopoulos MH, McBride AA (1998) Bovine papillomavirus type 1 genomes and the E2 transactivator protein are closely associated with mitotic chromatin. *J Virol* **72:** 2079-2088

Skyldberg B, Fujioka K, Hellstrom AC, Sylven L, Moberger B, Auer G (2001) Human papillomavirus infection, centrosome aberration, and genetic stability in cervical lesions. *Mod Pathol* **14:** 279-284

Slebos RJ, Kessis TD, Chen AW, Han SM, Hedrick L, Cho KR (1995) Functional consequences of directed mutations in human papillomavirus E6 proteins: abrogation of p53-mediated cell cycle arrest correlates with p53 binding and degradation in vitro. *Virology* **208:** 111-120

Smith-McCune K, Kalman D, Robbins C, Shivakumar S, Yuschenkoff L, Bishop JM (1999) Intranuclear localization of human papillomavirus 16 E7 during transformation and preferential binding of E7 to the Rb family member p130. *Proc Natl Acad Sci U S A* **96:** 6999-7004

Song S, Liem A, Miller JA, Lambert PF (2000) Human papillomavirus types 16 E6 and E7 contribute differently to carcinogenesis. *Virology* **267:** 141-150

Song S, Pitot HC, Lambert PF (1999) The human papillomavirus type 16 E6 gene alone is sufficient to induce carcinomas in transgenic animals. *J Virol* **73:** 5887-5893

Spruck CH, Strohmaier HM (2002) Seek and destroy: SCF ubiquitin ligases in mammalian cell cycle control. *Cell Cycle* **1:** 250-254

Stanley MA, Browne HM, Appleby M, Minson AC (1989) Properties of a non-tumorigenic human cervical keratinocyte cell line. *Int J Cancer* **43:** 672-676

Steger G, Corbach S (1997) Dose-dependent regulation of the early promoter of human papillomavirus type 18 by the viral E2 protein. *J Virol* **71:** 50-58

Steger G, Schnabel C, Schmidt HM (2002) The hinge region of the human papillomavirus type 8 E2 protein activates the human p21(WAF1/CIP1) promoter via interaction with Sp1. *J Gen Virol* **83:** 503-510

Stenlund A (2003) Initiation of DNA replication: Lessons from viral initiator proteins *Nature Reviews Molecular Cell Biology* **4:** 777-785

Sterlinko Grm H, Banks L (2004) HPV proteins as targets for therapeutic intervention. *Antiviral therapy* **9:** 665-678

Stoppler H, Stoppler MC, Johnson E, Simbulan-Rosenthal CM, Smulson ME, Iyer S, Rosenthal DS, Schlegel R (1998) The E7 protein of human papillomavirus type 16 sensitizes primary human keratinocytes to apoptosis. *Oncogene* **17:** 1207-1214

Storey A, Banks L (1993) Human papillomavirus type 16 E6 gene cooperates with EJ-ras to immortalize primary mouse cells. *Oncogene* **8:** 919-924

Storey A, Piccini A, Massimi P, Bouvard V, Banks L (1995) Mutations in the human papillomavirus type 16 E2 protein identify a region of the protein involved in binding to E1 protein. *J Gen Virol* **76 (Pt 4):** 819-826

Storey A, Pim D, Murray A, Osborn K, Banks L, Crawford L (1988) Comparison of the in vitro transforming activities of human papillomavirus types. *Embo J* **7:** 1815-1820

Storey A, Thomas M, Kalita A, Harwood C, Gardiol D, Mantovani F, Breuer J, Leigh IM, Matlashewski G, Banks L (1998) Role of a p53 polymorphism in the development of human papillomavirus-associated cancer. *Nature* **393:** 229-234

Straight SW, Herman B, McCance DJ (1995) The E5 oncoprotein of human papillomavirus type 16 inhibits the acidification of endosomes in human keratinocytes. *J Virol* **69:** 3185-3192

Strohmaier H, Spruck CH, Kaiser P, Won KA, Sangfelt O, Reed SI (2001) Human F-box protein hCdc4 targets cyclin E for proteolysis and is mutated in a breast cancer cell line. *Nature* **413:** 316-322

Suizu F, Ryo A, Wulf G, Lim J, Lu KP (2006) Pin1 regulates centrosome duplication, and its overexpression induces centrosome amplification, chromosome instability, and oncogenesis. *Mol Cell Biol* **26:** 1463-1479

Tan SH, Gloss B, Bernard HU (1992) During negative regulation of the human papillomavirus-16 E6 promoter, the viral E2 protein can displace Sp1 from a proximal promoter element. *Nucleic acids research* **20:** 251-256

Tan TM, Gloss B, Bernard HU, Ting RC (1994) Mechanism of translation of the bicistronic mRNA encoding human papillomavirus type 16 E6-E7 genes. *J Gen Virol* **75 (Pt 10):** 2663-2670

Thain A, Webster K, Emery D, Clarke AR, Gaston K (1997) DNA binding and bending by the human papillomavirus type 16 E2 protein. Recognition of an extended binding site. *J Biol Chem* **272:** 8236-8242

Thierry F, Benotmane MA, Demeret C, Mori M, Teissier S, Desaintes C (2004) A genomic approach reveals a novel mitotic pathway in papillomavirus carcinogenesis. *Cancer Res* **64:** 895-903

Thomas JT, Hubert WG, Ruesch MN, Laimins LA (1999a) Human papillomavirus type 31 oncoproteins E6 and E7 are required for the maintenance of episomes during the viral life cycle in normal human keratinocytes. *Proc Natl Acad Sci U S A* **96:** 8449-8454

Thomas JT, Laimins LA (1998) Human papillomavirus oncoproteins E6 and E7 independently abrogate the mitotic spindle checkpoint. *J Virol* **72:** 1131-1137

Thomas M, Banks L (1998) Inhibition of Bak-induced apoptosis by HPV-18 E6. *Oncogene* **17:** 2943-2954

Thomas M, Massimi P, Banks L (1996) HPV-18 E6 inhibits p53 DNA binding activity regardless of the oligomeric state of p53 or the exact p53 recognition sequence. *Oncogene* **13:** 471-480

Thomas M, Pim D, Banks L (1999b) The role of the E6-p53 interaction in the molecular pathogenesis of HPV. *Oncogene* **18:** 7690-7700

Thompson DA, Belinsky G, Chang TH, Jones DL, Schlegel R, Munger K (1997) The human papillomavirus-16 E6 oncoprotein decreases the vigilance of mitotic checkpoints. *Oncogene* **15:** 3025-3035

Thorland EC, Myers SL, Gostout BS, Smith DI (2003) Common fragile sites are preferential targets for HPV16 integrations in cervical tumors. *Oncogene* **22:** 1225-1237

Tong X, Howley PM (1997) The bovine papillomavirus E6 oncoprotein interacts with paxillin and disrupts the actin cytoskeleton. *Proc Natl Acad Sci U S A* **94:** 4412-4417

Tugizov S, Berline J, Herrera R, Penaranda ME, Nakagawa M, Palefsky J (2005) Inhibition of human papillomavirus type 16 E7 phosphorylation by the S100 MRP-8/14 protein complex. *J Virol* **79:** 1099-1112

Turnell AS, Mymryk JS (2006) Roles for the coactivators CBP and p300 and the APC/C E3 ubiquitin ligase in E1A-dependent cell transformation. *Br J Cancer* **95:** 555-560

Van Hoof C, Goris J (2004) PP2A fulfills its promises as tumor suppressor: which subunits are important? *Cancer cell* **5:** 105-106

Van Tine BA, Dao LD, Wu SY, Sonbuchner TM, Lin BY, Zou N, Chiang CM, Broker TR, Chow LT (2004) Human papillomavirus (HPV) origin-binding protein associates with mitotic spindles to enable viral DNA partitioning. *Proc Natl Acad Sci U S A* **101:** 4030-4035

Viejo-Borbolla A, Ottinger M, Bruning E, Burger A, Konig R, Kati E, Sheldon JA, Schulz TF (2005) Brd2/RING3 interacts with a chromatin-binding domain in the Kaposi's Sarcoma-associated herpesvirus latency-associated nuclear antigen 1 (LANA-1) that is required for multiple functions of LANA-1. *J Virol* **79:** 13618-13629

Vogelstein B, Kinzler KW (2004) Cancer genes and the pathways they control. *Nature medicine* **10:** 789-799

Vogt M, Butz K, Dymalla S, Semzow J, Hoppe-Seyler F (2006) Inhibition of Bax activity is crucial for the antiapoptotic function of the human papillomavirus E6 oncoprotein. *Oncogene* **25:** 4009-4015

Wang J, Sampath A, Raychaudhuri P, Bagchi S (2001) Both Rb and E7 are regulated by the ubiquitin proteasome pathway in HPV-containing cervical tumor cells. *Oncogene* **20:** 4740-4749

Wang Q, Griffin H, Southern S, Jackson D, Martin A, McIntosh P, Davy C, Masterson PJ, Walker PA, Laskey P, Omary MB, Doorbar J (2004) Functional analysis of the human papillomavirus type 16 E1=E4 protein provides a mechanism for in vivo and in vitro keratin filament reorganization. *J Virol* **78:** 821-833

Wang YW, Chang HS, Lin CH, Yu WC (2007) HPV-18 E7 conjugates to c-Myc and mediates its transcriptional activity. *The international journal of biochemistry & cell biology* **39:** 402-412

Watt FM (1998) Epidermal stem cells: markers, patterning and the control of stem cell fate. *Philosophical transactions of the Royal Society of London* **353:** 831-837

Watt FM, Lo Celso C, Silva-Vargas V (2006) Epidermal stem cells: an update. *Current opinion in genetics & development* **16:** 518-524

Weber JD, Kuo ML, Bothner B, DiGiammarino EL, Kriwacki RW, Roussel MF, Sherr CJ (2000) Cooperative signals governing ARF-mdm2 interaction and nucleolar localization of the complex. *Mol Cell Biol* **20:** 2517-2528

Webster K, Parish J, Pandya M, Stern PL, Clarke AR, Gaston K (2000) The human papillomavirus (HPV) 16 E2 protein induces apoptosis in the absence of other HPV proteins and via a p53-dependent pathway. *J Biol Chem* **275:** 87-94

Wells SI, Aronow BJ, Wise TM, Williams SS, Couget JA, Howley PM (2003) Transcriptome signature of irreversible senescence in human papillomavirus-positive cervical cancer cells. *Proc Natl Acad Sci U S A* **100:** 7093-7098

Westbrook TF, Nguyen DX, Thrash BR, McCance DJ (2002) E7 abolishes raf-induced arrest via mislocalization of p21(Cip1). *Mol Cell Biol* **22:** 7041-7052

Wilson R, Fehrmann F, Laimins LA (2005) Role of the E1--E4 protein in the differentiation-dependent life cycle of human papillomavirus type 31. *J Virol* **79:** 6732-6740

Wilson R, Ryan GB, Knight GL, Laimins LA, Roberts S (2007) The full-length E1E4 protein of human papillomavirus type 18 modulates differentiation-dependent viral DNA amplification and late gene expression. *Virology* **362:** 453-460

Wu H, Ceccarelli DF, Frappier L (2000) The DNA segregation mechanism of Epstein-Barr virus nuclear antigen 1. *EMBO reports* **1:** 140-144

Wulf G, Finn G, Suizu F, Lu KP (2005) Phosphorylation-specific prolyl isomerization: is there an underlying theme? *Nature cell biology* **7:** 435-441

Ying H, Xiao ZX (2006) Targeting retinoblastoma protein for degradation by proteasomes. *Cell Cycle* **5:** 506-508

Yoshinouchi M, Yamada T, Kizaki M, Fen J, Koseki T, Ikeda Y, Nishihara T, Yamato K (2003) In vitro and in vivo growth suppression of human papillomavirus 16-positive cervical cancer cells by E6 siRNA. *Mol Ther* **8:** 762-768

You J, Croyle JL, Nishimura A, Ozato K, Howley PM (2004) Interaction of the bovine papillomavirus E2 protein with Brd4 tethers the viral DNA to host mitotic chromosomes. *Cell* **117:** 349-360

Yu T, Peng YC, Androphy EJ (2007) Mitotic kinesin-like protein 2 binds and colocalizes with papillomavirus E2 during mitosis. *J Virol* **81:** 1736-1745

Yukawa K, Butz K, Yasui T, Kikutani H, Hoppe-Seyler F (1996) Regulation of human papillomavirus transcription by the differentiation-dependent epithelial factor Epoc-1/skn-1a. *J Virol* **70:** 10-16

Zerfass K, Schulze A, Spitkovsky D, Friedman V, Henglein B, Jansen-Durr P (1995) Sequential activation of cyclin E and cyclin A gene expression by human papillomavirus type 16 E7 through sequences necessary for transformation. *J Virol* **69:** 6389-6399

Zhang B, Chen W, Roman A (2006) The E7 proteins of low- and high-risk human papillomaviruses share the ability to target the pRB family member p130 for degradation. *Proc Natl Acad Sci U S A* **103:** 437-442

Zhang B, Laribee RN, Klemsz MJ, Roman A (2004) Human papillomavirus type 16 E7 protein increases acetylation of histone H3 in human foreskin keratinocytes. *Virology* **329:** 189-198

Zhang B, Li P, Wang E, Brahmi Z, Dunn KW, Blum JS, Roman A (2003) The E5 protein of human papillomavirus type 16 perturbs MHC class II antigen maturation in human foreskin keratinocytes treated with interferon-gamma. *Virology* **310:** 100-108

Zhang B, Spandau DF, Roman A (2002) E5 protein of human papillomavirus type 16 protects human foreskin keratinocytes from UV B-irradiation-induced apoptosis. *J Virol* **76:** 220-231

Zheng ZM, Baker CC (2006) Papillomavirus genome structure, expression, and post-transcriptional regulation. *Front Biosci* **11:** 2286-2302

Zhou BP, Liao Y, Xia W, Spohn B, Lee MH, Hung MC (2001) Cytoplasmic localization of p21Cip1/WAF1 by Akt-induced phosphorylation in HER-2/neu-overexpressing cells. *Nature cell biology* **3:** 245-252

Zimmermann H, Degenkolbe R, Bernard HU, O'Connor MJ (1999) The human papillomavirus type 16 E6 oncoprotein can down-regulate p53 activity by targeting the transcriptional coactivator CBP/p300. *J Virol* **73:** 6209-6219

Zou N, Lin BY, Duan F, Lee KY, Jin G, Guan R, Yao G, Lefkowitz EJ, Broker TR, Chow LT (2000) The hinge of the human papillomavirus type 11 E2 protein contains major determinants for nuclear localization and nuclear matrix association. *J Virol* **74:** 3761-3770

Zou N, Liu JS, Kuo SR, Broker TR, Chow LT (1998) The carboxyl-terminal region of the human papillomavirus type 16 E1 protein determines E2 protein specificity during DNA replication. *J Virol* **72:** 3436-3441

Zwerschke W, Joswig S, Jansen-Durr P (1996) Identification of domains required for transcriptional activation and protein dimerization in the human papillomavirus type-16 E7 protein. *Oncogene* **12:** 213-220

Zwerschke W, Mazurek S, Massimi P, Banks L, Eigenbrodt E, Jansen-Durr P (1999) Modulation of type M2 pyruvate kinase activity by the human papillomavirus type 16 E7 oncoprotein. *Proc Natl Acad Sci U S A* **96:** 1291-1296